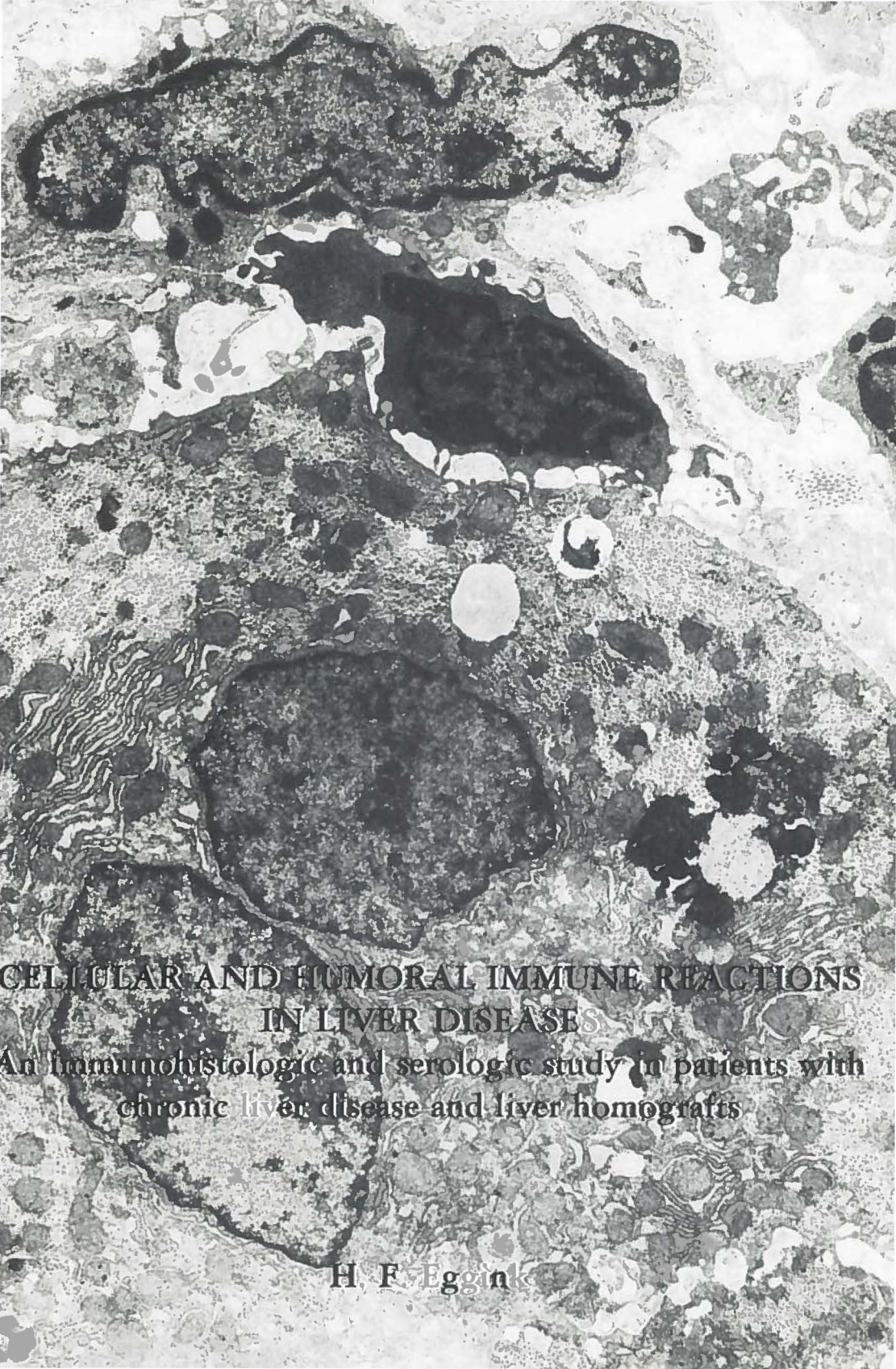

CELLULAR AND HUMORAL IMMUNE REACTIONS IN LIVER DISEASE

An immunohistologic and serologic study in patients with chronic liver disease and liver homografts

H F Eggink

CELLULAR AND HUMORAL IMMUNE REACTIONS IN LIVER DISEASES

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Stellingen

I.

Lever membraan autoantilichamen (LMA) spelen geen oorzakelijke rol bij het ontstaan van leverziekten.

II.

Het opnieuw verschijnen van autoantilichamen in het serum van patiënten na levertransplantatie, mag niet als argument gebruikt worden voor het terugkeren van de oorspronkelijke leverziekte.

III.

Voor het bepalen van het klinisch beleid na levertransplantatie zijn leverbiopsieën van essentieel belang.

IV.

Bij een patiënt, verdacht voor een niet-gemetastaseerd primair hepatocellulair carcinoom, is een leverbiopsie gecontraïndiceerd, aangezien hierna levertransplantatie als vorm van curatieve therapie is uitgesloten.

V.

Voor de beoordeling en interpretatie van een leverbiopsie kan men in het algemeen volstaan met de normale routine histologische kleuringen.

VI.

Voor het karakteriseren van het ontstekingsinfiltraat in weefsels moeten meerdere monoclonale antilichamen naast elkaar worden gebruikt, ten einde het type ontstekingscel zo goed mogelijk vast te leggen.

VII.

Het aantonen van een chlamydia infektie met behulp van monoclonale antilichamen in routine cervixstrijkjes is gemakkelijk uitvoerbaar en werkt kostenbesparend.

VIII.

Bij patiënten met diabetes mellitus dient men bij het voorschrijven van een eiwitrijk dieet, ter suppletie van de kalorieënbehoefte, rekening te houden met de daarmee verhoogde kans op het ontwikkelen van diabetische glomerulopathie.

IX.

Slechts bij een zeer duidelijke indikatiestelling kan een schildklierpunktie van diagnostische waarde zijn.

X.

Het nicotinegehalte in het bloed van een roker wordt bepaald door het aantal sigaretten per dag en niet door het soort sigaret (Benowitz et al, N Eng J Med 1983;309:139-142).

XI.

In een tijd waarin kwaadaardige aandoeningen volgens vaste protocollen worden behandeld, zou ook het postmortale onderzoek, ter evaluatie van de behandeling, in dergelijke protocollen moeten worden opgenomen.

XII.

De verlenging van de opleiding tot patholoog-anatoom met één jaar, zou gedeeltelijk gebruikt moeten worden voor een stage in de periferie.

XIII.

Indien een lymfeklier wordt verwijderd voor diagnostische doeleinden, moet het insturen op formaline, zonder vooroverleg met de patholoog-anatoom, als een kunstfout worden beschouwd.

XIV.

Het windsurfen op de Waddenzee zonder volgboot zou verboden moeten worden.

Stellingen
behorende bij het proefschrift van

H. F. Eggink

CELLULAR AND HUMORAL IMMUNE REACTIONS
IN LIVER DISEASES
An immunohistologic and serologic study in patients with
chronic liver disease and liver homografts

Groningen, 10 oktober 1984

RIJKSUNIVERSITEIT TE GRONINGEN

CELLULAR AND HUMORAL IMMUNE REACTIONS IN LIVER DISEASES

An immunohistologic and serologic study in patients with chronic liver disease and liver homografts

PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de Rector Magnificus Dr. E. Bleumink
in het openbaar te verdedigen op woensdag 10 oktober 1984
des namiddags te 4.00 uur
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HENDRIK FREDERIK EGGINK

geboren te Groningen

1984
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GRONINGEN

Promotores : Prof. Dr. H. J. Houthoff
Prof. Dr. C. H. Gips
Prof. Dr. M. J. Hardonk
Referent : Dr. S. Poppema

To Truus
Carolien
Mark
Annemieke

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CONTENTS

ABBREVIATIONS

ADCC	antibody dependent cell-mediated cytotoxicity
AH	acute hepatitis
AHB	acute hepatitis B
ALD	alcoholic liver disease
AMA	anti-mitochondrial antibodies
ANA	anti-nuclear antibodies
autoAb	autoantibodies
CAH-B	chronic active hepatitis B
CALD	chronic active liver disease
CMC	cell-mediated cytotoxicity
CMV	cytomegalovirus
CMV-EA	cytomegalovirus early antigen
CMV-LA	cytomegalovirus late antigen
CPH-B	chronic persistent hepatitis B
DAB	diaminobenzidin
EM	electron microscopy
FDP	fibrin degradation products
FM	fibrin monomers
HBsAg	hepatitis B surface antigen
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HB-CALD	hepatitis B chronic active liver disease
HBV	hepatitis B virus
HSV	herpes simplex virus
IA-CALD	idiopathic autoimmune chronic active liver disease
IF	immunofluorescence
Ig	immunoglobulins
IP	immunoperoxidase
K-cell	killer cell
LM	light microscopy
LM-Ag	liver membrane antigen
LMA	liver membrane autoantibodies
LSP	liver specific protein
MoAbs	monoclonal antibodies
NK-cell	natural killer cell
OLT	orthotopic liver transplantation

PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PMN	piecemeal necrosis
PT	portal tract
SMA	smooth muscle antibodies
SEM	scanning electron microscopy
TEM	transmission electron microscopy

CHAPTER 1

Introduction

A chronic inflammation of the liver, known as chronic active liver disease (CALD) or chronic hepatitis, is a condition interfering with the balanced functions of liver cells and the liver as a whole. It may be a stationary condition over long periods of time or progressively lead to liver cirrhosis and/or liver failure, ultimately leading to the patient's death. Various histologic forms of chronic hepatitis have been described in relation to the activity of the inflammation and have therefore been linked to the progression of morphologic and functional disturbances. One form of chronic hepatitis is chronic active hepatitis (CAH), characterized by an inflammatory infiltrate in the portal connective tissue invading the adjacent liver parenchyma and here surrounding individual liver cells that eventually degenerate and become necrotic. The desintegration of individual periportal liver cells surrounded by inflammatory cells is known as piecemeal necrosis (PMN), which represents the common histologic hallmark of CAH (1, 2). CAH may occur as a result of various aetiologic factors, both exogenous and endogenous. These include chronic viral infections of the liver, metabolic diseases, primary gastrointestinal diseases with liver involvement, autoimmune diseases, allergic drug reactions and host reactions to liver homografts. The common histologic hallmark of PMN has suggested the possibility that an identical pathogenetic mechanism might underly the liver cell damage in all these disease states causing CAH (3). On the other hand, the heterogeneity of exogenous and endogenous aetiologic factors and the different reactions to therapeutic regimens in relation to the aetiology of CAH have suggested that the always similar morphology of PMN is the expression of various pathogenetic mechanisms.

A main and useful partition of the various aetiologic/pathogenetic factors in CAH could be to distinguish between the conditions with primary changes in liver cells, that as a secondary reaction attract inflammatory cells, and those conditions with as primary change an imbalanced reaction of the immune apparatus towards liver parenchyma. The latter conditions include autoimmune diseases (as for instance idiopathic autoimmune CALD: IA-CALD) and rejection phenomena to liver homografts: in these conditions immunosuppressive therapy usually may be beneficial (4). The conditions with primary involvement of the liver cells include chronic viral infections

of the liver (as for instance in chronic hepatitis B virus infection: HB-CALD) and metabolic disorders (as for instance in Wilson's disease, affecting copper metabolism); here, immunosuppressive therapy has no clear beneficial effect and may be even deleterious (5, 6). However, in both these groups of CAH immune mechanisms are involved in the pathogenesis, whether as cause or as effect, and in both groups lymphocytes and plasma cells occur in the inflammatory infiltrate in the liver and autoantibodies may occur in the serum as a reflection of the immune mechanisms involved.

Several lines of evidence have further substantiated the contribution of immune mechanisms to the pathogenesis of CALD. In vitro studies have demonstrated that the peripheral blood lymphocytes of patients with IA-CALD, HB-CALD or primary biliary cirrhosis (PBC, another autoimmune liver disease) are cytotoxic for heterologous (7), homologous (8) and autologous (9) liver cells and that these lymphocytes manifest increased reactivity when exposed in vitro to hepatic, biliary and extra-hepatic antigens (10-12). Also, in the serum of patients with CALD several types of liver-specific autoantibodies may be present which have been shown by *in vitro* methods to induce liver cell damage in cooperation with lymphocytes (13).

The classical concept of the immune system consisting of T lymphocytes, B lymphocytes, plasma cells and cells of the mononuclear/macrophage system has been expanded by the discovery of various subsets of populations of morphologic identical cells. These subsets can be defined by mutual differences in antigenic surface markers and functional characteristics, these two not necessarily completely coinciding. The balance between effector and regulatory subsets governs the outcome of antigenic stimulation and is critical for immune homeostasis (14). The subset of inducer T cells is central for the activation of other T cells, B cells and macrophages. This inductive influence is regulated by the presence of feedback mechanisms including suppressor T cell subsets, that function to inactivate the inducer cell subset or the effector cell subset itself. Functional loss or activation of lymphocyte subsets may each lead to a variety of immunologic disorders including immunodeficiency or autoimmune diseases (15-17).

The aim of this study was to unravel the immune mechanism involved in the various forms of CALD of different aetiology. To this purpose we studied the presence and contribution of cellular and humoral immune factors in sections of liver biopsies and of humoral factors in serum samples. Recently developed monoclonal antibodies to differentiation antigens on human

lymphocytes allowed for the examination of the phenotype of lymphocyte subsets (18-21). These methods are applied to liver biopsy material of carefully selected patient groups with untreated IA-CALD, HB-CALD and PBC, respectively (*chapter 2*). Special emphasis is given to differences in lymphocyte subsets according to aetiology and to the relation between lymphocytes and degenerating liver cells, in order to define the phenotype of the potential effector cell subset in the presumed type of cytotoxic reaction. In hepatitis B viral infection of the liver these relations are further clarified in patients groups of acute and chronic infection, by comparing the distribution of viral antigens in liver cells with the type and distribution of potential effector cell subsets (*chapter 3*). As the expression of a single differentiation antigen may be shared by more than one lymphocyte subset, phenotyping with only one or a small number of monoclonal antibodies may not give a reliable picture of the lymphocyte subsets involved. For a pathogenetic interpretation, the ultimate goal of relating phenotype to function is best approached by the application of a panel of monoclonals to define the various lymphocyte subsets involved (*chapter 3: addendum*). The availability of graft biopsies from liver transplant recipients enabled us to study immune reactions in the liver from quite another angle. In order to do so, the histopathology and differential diagnosis of the major graft syndromes has to be unraveled first (*chapter 4*), as literature data was rather scanty and mainly based on autopsy material. In episodes of acute rejection, with a morphology resembling CALD, the relation between the participating lymphocyte subsets and liver cell necrosis is studied and compared with IA-CALD and PBC (*chapter 5*). In the chapters mentioned above the possible role of humoral factors is evaluated by the study of the in situ presence of immunoglobulins in liver tissue, in *chapter 6* liver membrane autoantibodies (LMA) in the serum are studied. The presumed role of LMA in the pathogenesis of CALD is investigated by comparison of its presence in serum with the phenotype of potential effector cell subsets in the liver. Finally, in *chapter 7* the results of this study are discussed in relation to the literature and possibilities for future research in the pathogenesis of CALD are indicated.

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CHAPTER 2

Cellular and humoral immune reactions in chronic active liver disease. I. Lymphocyte subsets in liver biopsies of patients with untreated idiopathic autoimmune hepatitis, chronic active hepatitis B and primary biliary cirrhosis*

H. F. Eggink, H. J. Houthoff, Sippie Huitema, C. H. Gips[1] & S. Poppema

Departments of Pathology and [1]Medicine, University Hospital and University of Groningen, Groningen, The Netherlands.

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Summary

In liver biopsies of 37 patients with chronic active liver disease (CALD) the inflammatory infiltrate was studied with monoclonal antibodies to the surface antigens on helper/inducer (OKT4+), suppressor/cytotoxic (OKT8+), killer/natural killer (OKM1,2+) cells and common T cell antigens (OKT1+, OKT3+). Furthermore OKT11 antibody was applied, which defines the E rosette receptor. Special emphasis was given to areas with piece-meal necrosis (PMN). In areas with PMN in idiopathic autoimmune CALD (IA-CALD, $n = 15$) OKT8+ and OKM+ lymphocytes and IgG plasma cells were present, whereas in hepatitis B-CALD (HB-CALD, $n = 12$) almost exclusively OKT8+ cells were found. In PBC ($n = 10$) OKT4+ cells in central parts of portal tracts and OKT8+ cells in areas with PMN predominated. These findings indicate that in IA-CALD antibody-dependent cell-mediated cytotoxicity (ADCC), as well as T cell cytotoxicity may be responsible for liver cell damage, while in HB-CALD T cell cytotoxicity seems to be the only mechanism. In PBC liver cell damage also predominantly is the result of T cell cytotoxicity. In addition, helper T lymphocytes seem to play a role since these are found in central areas of the portal tracts.

INTRODUCTION

Chronic active liver disease (CALD) is a syndrome of multifactorial aetiology characterized by continuous and progressive inflammation of the liver and liver cell destruction. The histological hallmark of CALD is an inflammatory infiltrate of predominantly lymphocytes and plasma cells in a portal tract mesenchyme, invading the periportal liver parenchyma with necrosis of individual cells in this periportal area, the latter generally known

as 'piece-meal necrosis' (PMN) (Groote et al., 1968; Scheuer, 1977). CALD can not be considered a disease entity, as it is of multifactorial aetiology with clinical and serological findings and optimal therapeutical regimens that may vary according to the aetiology. Main groups according to aetiology are (1) CALD following type B viral hepatitis with generally viral surface antigen (HBsAg) present in the serum (HB-CALD) and (2) CALD of unknown cause with high levels of abnormal serum antibodies (idiopathic autoimmune type: IA-CALD). Primary biliary cirrhosis (PBC) is sometimes included as it shares many features, including the lymphocytic infiltration and PMN, with CALD. Despite this various aetiology, the common histological hallmark of lymphocytic infiltration and PMN, raised immunoglobulin levels and abnormal antibodies in the serum, all support the idea of immunological factors in the pathogenesis of CALD and it has been suggested that lymphocytes surrounding the affected liver cells represent a common immunological effector mechanism (Groote et al., 1968; Jensen et al., 1978).

Recently, a series of monoclonal antibodies reactive with human thymocytes and peripheral T cell antigens have been developed and found to be specific for functionally distinct subclasses of T lymphocytes (Reinherz et al., 1979, 1980; Kung et al., 1979). These antibodies allow for a precise characterization of lymphocytes of T cell lineage in suspension, but also in tissue sections (Bhan et al., 1980; Poppema et al., 1981). In a previous study we demonstrated the feasibility of T cell subset staining in frozen sections of liver biopsies (Eggink et al., 1981), in the present study liver biopsies of non-treated patients with HB-CALD, IA-CALD or PBC have been investigated with these methods. Special emphasis has been given to answer the following questions: (1) which subsets of T and B lymphocytes are present in the liver during CALD and are there differences in lymphocyte subsets according to aetiology, (2) which subsets are especially related to PMN and might thus represent the effector cell in any kind of cytotoxic reaction as the basis of CALD.

MATERIALS AND METHODS

Patient groups and materials. Liver biopsies of three groups of patients with CALD were studied. The first group consisted of patients ($n = 15$, 12 female, 3 male, age 16-78 years) with hypergammaglobulinaemia, high titre serum autoantibodies to smooth muscle (SMA) and nuclei (ANA), no detectable HBsAg or antibodies to core antigen (anti-HBc) in the serum, no

history of blood transfusion and no epidemiological data suggestive of a non-A non -B hepatitis virus infection. A clinical diagnosis of idiopathic autoimmune liver disease was made: the IA-CALD group. The patients of the second group (n = 12, 2 female, 10 male, age 25-47 years) were all HBsAg seropositive with anti-HBc serum antibodies and a clinical diagnosis of chronic active hepatitis B: the HB-CALD group. The patients of the third group (n = 10, 9 female, 1 male, age 46-80 years) had anti-mitochondrial antibodies (AMA), increased IgM levels and elevated alkaline phosphatase values in the serum, the patients' histories included intermittent periods of itching. A clinical diagnosis of primary biliary cirrhosis was made: the PBC group.

Percutaneous liver biopsies were performed using a 1·6 mm diameter Menghini needle. A small part of the biopsy was immediately frozen in Freon-22 and stored at —70°C. The larger part of the biopsy was fixed in 4% formaldehyde, embedded in paraplast and used for light microscopy and immunoperoxidase studies. From five patients with PBC and three with IA-CALD larger and multiple blocks of liver tissue could be examined, since the liver of these patients was removed during orthotopic liver transplantation.

Lymphocyte subsets. The lymphocyte subsets were characterized with hybridoma-produced, monoclonal antibodies which were obtained from Ortho Pharmaceutical Corp. (Raritan, New Jersey, USA). The production, growth and characterization of these antibodies have been the subject of a series of recent reports. Literature data about the characteristics of lymphocyte subsets as determined by these antibodies are summarized in Table 1.

To demonstrate reactivity of these lymphocyte subsets in tissue sections with monoclonal antibodies an indirect immunoperoxidase technique was used. In short, 6 μm thick frozen sections were air dried with a ventilator for 30 min. The sections were fixed in acetone for 10 min at room temperature and shortly washed in phosphate-buffered saline (PBS), pH 7·4. The sections were incubated with 25 μl of diluted antibody for 30 min. In addition, sections were incubated with control ascites or PBS. All sections were subsequently incubated with peroxidase conjugated rabbit anti-mouse Ig serum (Dakopatts, Copenhagen, Denmark), diluted 1 : 20 for 15 min, supplemented with 1% human AB serum. Between incubations the sections were washed for 10 min in three changes of PBS. The sections were stained with 3-amino-9-ethylcarbazole and H_2O_2 in 0·1 M acetate buffer, pH 4·9 for 5-10 min to develop a red reaction product (Graham, Lundholm & Karnovsky,

Table 1. Reactivity of monoclonal antibodies with human thymocytes and peripheral blood monocytes.

Antiserum	Lymphocyte subsets positive with antiserum	References
OKT 1, OKT 3	100% of peripheral T cells and 10% of thymocytes	Reinherz et al. (1980, 1981)
OKT 4	55% of peripheral T cells, defines inducer/helper T cells	Reinherz et al. (1980, 1981)
OKT 6	0% of peripheral T cells, defines immature, thymocyte-like subset	Reinherz et al. (1980, 1981)
OKT 8	30% of peripheral T cells, defines suppressor/cytotoxic T cells	Reinherz et al. (1980, 1981)
OKT 11	all E rosette receptor bearing cells: 100% of peripheral T cells and a population of non-T cells with natural killer and killer activities	Verbi et al. (1982)
OKM 1, OKM 2	monocytes, polymorphonuclear leucocytes and a population of non-T non-B lymphocyte-like cells with natural killer and/or killer activity	Kay & Horwitz (1980) Breard et al. (1980)

1965). The reaction was terminated by washing in acetate buffer. The sections were air dried and cover slipped with Aquamount (Gurr, Essex, England).

B lymphocytes, plasma cells and immunoglobulins. For the demonstration of B lymphocytes 6 μm thick cryostat sections of frozen tissue were stained with the unconjugated peroxidase technique for the demonstration of surface IgM. The first step consisted of rabbit anti-human IgM serum diluted 1 : 200, followed by incubation with swine anti-rabbit Ig serum diluted 1 : 50 and finally peroxidase rabbit anti-peroxidase complexes (Dako, Copenhagen, Denmark), diluted 1 : 50. The staining procedure for peroxidase was the same as mentioned above. Plasma cells and immunoglobulins on the surface of liver cells were demonstrated in 4 μm thick paraffin sections. After pre-incubation with normal rabbit serum diluted 1 : 5 at room temperature for 10 min, sections were incubated with peroxidase conjugated rabbit anti-human IgG, IgM or IgA serum (Dako, Copenhagen, Denmark) for 60 min at 37°C. These sections were stained with diaminobenzidin (DAB) for 5 min. For every technique, control sections were incubated with normal serum or PBS in the first step.

RESULTS

For each group of liver biopsies the results will be given with regard to the following questions: (1) is the inflammatory infiltrate predominantly of T cell, of B cell, or of non-T, non-B cell origin?, (2) what are the subclasses of the T and B lymphocytes in the infiltrate and how are they located with respect to portal tracts, parenchyma and periportal areas? and (3) which cells can be found predominantly or exclusively in association with hepatocytes with PMN.

In general, hepatocytes and portal tract structures were negative with all methods, apart from some immunoglobulin staining of the portal vessels and mesenchyme. The Kupffer cells and endothelial lining cells of the liver sinusoids were also negative with all methods, especially also with the OKM antibodies. No OKT6+ lymphocytes were found in any of the cases.

IA-CALD

In IA-CALD a majority of the lymphocytes were OKT1+,3+ and thus of T cell origin. However, in this group in nine out of 15 cases clearly more lymphocytes were stained with OKT11 than with OKT1 and OKT3. These OKT1−,3−,11+ lymphocytes were especially obvious in areas with PMN and throughout the parenchyma. OKT8+ lymphocytes clearly predominated in portal tracts and in periportal areas with PMN (Fig. 1a), whereas OKT4+ lymphocytes were only present in small numbers mostly in the central parts of portal tracts. In most liver biopsies, a number of lymphocyte-like cells in the liver sinusoids were OKT1−,3− but appeared to be positive with OKT11 and also with OKT8 antibodies. OKM+ lymphocyte-like cells were seen in 13 out of 15 cases in areas with PMN (Fig. 1b) and scattered throughout the parenchyma and portal tracts. The amount of OKM+ lymphocyte-like cells was far less than the amount of OKT8+ lymphocytes. Considerable numbers of polymorphonuclear leucocytes were present, especially in areas with PMN.

Very few B lymphocytes were found, except for one case with a moderate amount of B lymphocytes in portal tracts. Plasma cells with IgG and IgA were present in most cases and often in areas with PMN, the IgG positive plasma cells clearly predominated (Fig. 1c). Plasma cells with IgM were absent except for one case, where many plasma cells with IgG, IgA and IgM were found in areas with PMN. Immunoglobulins at the surface of liver cells were only seen in one case and not in relation to areas with PMN.

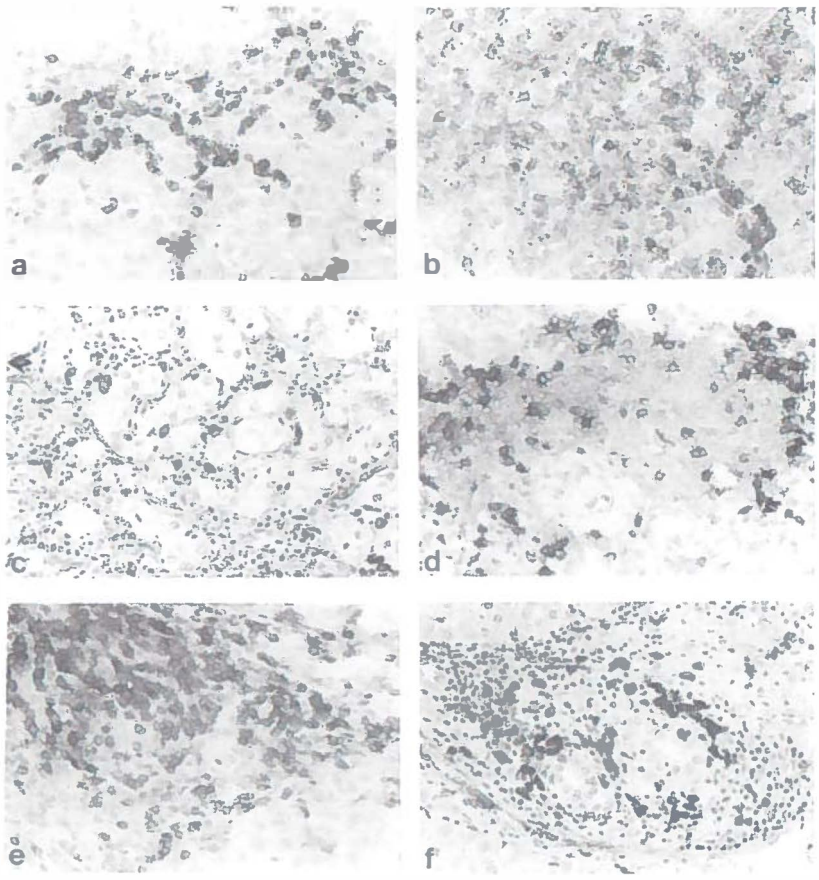

Fig. 1. Immunoperoxidase staining of liver biopsy sections with monoclonal anti-lymphocyte antibodies and anti-immunoglobulin sera. (a,b,c) Idiopathic autoimmune chronic active liver disease. OKT8+ cytotoxic/suppressor lymphocytes (a), OKM+ lymphocyte-like cells (b) and IgG positive plasma cells (c) in areas with piece-meal necrosis. (× 160). (d,e,f,) Primary biliary cirrhosis. OKT8+ cytotoxic/suppressor lymphocytes in periportal areas with piece-meal necrosis and negative lymphocytes in central part of portal tract (d), OKT4+ helper/inducer lymphocytes in central part of portal tract (e) and IgM positive plasma cells in portal tract around bile ducts (f). (× 100).

HB-CALD

In HB-CALD a majority of the lymphocytes were OKT1+,3+ and thus of T cell origin. In fact, only very few or no B lymphocytes were found in these biopsies. OKT11 gave similar results as OKT1 and OKT3. About equal

numbers of OKT4+ and OKT8+ lymphocytes were present in portal tracts, in areas with PMN the OKT8+ cells clearly predominated. In six cases, OKM+ lymphocyte-like cells were present in small numbers throughout the parenchyma; however, these cells were absent in portal tracts and in areas with PMN. Moderate numbers of polymorphonuclear leucocytes were present in all cases without any special predilection site.

Plasma cells with IgG and IgA were scanty in most cases, plasma cells with IgM were absent. The plasma cells were mostly found in portal tracts, sometimes also in areas with PMN. Immunoglobulins at liver cell membranes were present in five cases, mostly randomly distributed throughout the parenchyma and not especially related to areas with PMN.

PBC

Also in PBC a majority of the lymphocytes was OKT1+,3+ and thus of T cell origin. Staining with OKT11 generally gave results that were comparable to OKT1 and OKT3, in the liver parenchyma the OKT11+ cells clearly outnumbered the OKT1+,3+ cells, however. In the portal tracts both OKT4+ and OKT8+ lymphocytes were present; OKT4+ cells mostly in the central parts of the portal tracts and OKT8+ cells more to the periphery and in the adjoining areas with PMN (Fig. 1d,e). The sum of OKT4+ and OKT8+ cells clearly outnumbered the OKT1+,3+ cells. OKM+ lymphocyte-like cells were present in relatively small numbers in the liver parenchyma in eight cases, in six of these few cells were also present in the portal tracts. In only one case OKM+ cells were present in areas with PMN. Polymorphonuclear leucocytes were present in all cases; they occurred scattered throughout the parenchyma and were prominent in the peripheral areas of portal tracts. A moderate number of B lymphocytes was present in the portal tracts of two cases, in all other cases only few scattered B lymphocytes were present in the portal tracts. B lymphocytes in the parenchyma or in areas with PMN were not found. Comparable numbers of plasma cells with IgG and IgM were usually present in portal tracts and areas with PMN, in four cases the plasma cells with IgM predominated. There was a tendency for plasma cells with IgM to cluster around the persistent bile ducts (Fig. 1f). A smaller number of plasma cells with IgA was similarly present in all cases and mainly in portal tracts. Immunoglobulin localization at liver cell membranes was observed in five cases, a relation to areas with PMN was lacking.

Histology of the livers that were removed during orthotopic liver transplantation showed the presence of areas with ductural proliferation and

PMN (stage 2), portal fibrosis (stage 3) and liver cirrhosis (stage 4) in all cases. The study of larger sections, sampled from different areas in these livers showed a similar distribution of the lymphocyte subsets, providing an extra argument for the idea, that liver biopsies yielded representative results.

DISCUSSION

In this study we have determined the nature of lymphocyte subsets in the livers of three groups of patients with CALD, using frozen liver sections and immunoperoxidase methods. It has the obvious advantage that the lymphocyte populations in the liver itself are studied, as these populations are not necessarily the same as those in peripheral blood. This is exemplified by the increased numbers of OKT8+ lymphocytes in the livers from patients with CALD, as opposed to the reported decrease in suppressor cell function of peripheral blood lymphocytes (Tremolda et al., 1980; Rowen, Zetterman & Woltjen, 1980; Nonomura et al., 1982). Furthermore, it enables the identification of the lymphocyte subsets that are predominantly or exclusively involved in liver cell destruction by their location in areas with PMN.

The lymphocyte subsets as characterized by the monoclonal antibodies showed differences in prominence and/or distribution throughout the liver in relation to the three groups, the variability within each group was comparably minor. The OKT4+ subset defines the T helper cell population; these cells were present in relatively small numbers in IA-CALD and HB-CALD but prominent in PBC, predominantly in the central parts of portal tracts. Functions of T helper cells include the induction of B cell differentiation into immunoglobulin producing plasma cells and also of T-cell differentiation into cytotoxic T cells (Reinherz & Schlossman, 1980). The presence of many helper cells in PBC might be related to the presence of many plasma cells in the portal tracts. Also, helper cells are commonly present in relation to granuloma formation in various diseases i.e. as in sarcoidosis (Hunninghake & Crystal, 1981) and might thus be involved in the granulomatous destructive cholangitis of PBC. However, it can not be ruled out that the presence of many helper cells in PBC is only a reflection of the subset distribution in peripheral blood lymphocytes. A recent study (Bhan et al., 1982) on T cell subsets in peripheral blood of patients with PBC showed a reduced helper T cell population in early stages, but reduced suppressor/cytotoxic T cell population in advanced stages of the disease.

The reduced percentages of T lymphocytes in the peripheral blood may reflect migration of T lymphocytes to the diseased liver. The apparent extra diminution of suppressor/cytotoxic T lymphocytes in advanced stages is in agreement with our findings, since all our patients were stage II, III and IV showing PMN and extensive infiltration by OKT8 positive suppressor/cytotoxic T lymphocytes.

The OKT8+ subset comprises the cytotoxic/suppressor T cell population; these cells were prominent in the livers of all three groups and predominated in the peripheral parts of portal tracts and especially in areas with PMN. Though it is not possibile to differentiate between cytotoxic and suppressor T cells by the OKT8 antibody, it may be assumed that the presence of OKT8+ cells in areas with PMN reflects a cytotoxic rather than a suppressor functional activity. Accordingly, increased cytotoxic activity of peripheral blood lymphocytes against Chang cells has been found in patients with CALD (Wands & Isselbacher, 1975); however, natural killer as well as killer (ADCC) cell activity may be responsible for these findings (Serdengecti et al., 1981). Since OKT11+, as well as OKT8+ cells, clearly outnumbered the OKT1+, OKT3+ cells, especially in areas with PMN and throughout the parenchyma in IA-CALD and to a lesser extent in the parenchyma in PBC, it may be concluded that a population of E rosette positive OKT8+, but OKT1−, OKT3−, lymphocytes is present.

OKM1 and OKM2 react with monocytes, polymorphonuclear leucocytes and lymphocyte-like non-T, non-B cells with natural killer and killer activity (Breard et al., 1980). It is remarkable that Kupffer cells were negative with the OKM antibodies. In this repect, Kupffer cells are similar to other tissue macrophages, which also do not react with the OKM antibodies (Poppema et al., 1982). Polymorphonuclear leucocytes could readily be distinguished by their morphology, they occurred in all cases but were especially prominent in IA-CALD in areas with PMN. Also, the lymphocyte-like OKM *2* cells were most prominent in IA-CALD in areas with PMN, but also occurred scattered throughout the parenchyma in many cases of all three groups. Since OKM antibodies stain E rosette negative as well as E rosette positive cells and do not react with OKT1+,3+ or OKT8+ cells (Kay & Horwitz, 1980), it is plausible that some of the OKM+ natural killer and/or killer cells are also staining with the OKT11 antibodies.

B lymphocytes were inconspicuous in nearly all cases, and thus they are seemingly unimportant for the pathogenesis of liver disease in all three groups. Plasma cells were an inconspicuous feature in HB-CALD but were

prominent in IA-CALD and PBC. Based on cell numbers and distribution, the assumption of a relation between IgG producing plasma cells and OKM+ and/or OKT8+ lymphocytes in areas with PMN in IA-CALD seems justified. In PBC, a relation between the IgM producing plasma cells and the OKT4+ helper cells in the portal tracts might be assumed on the same ground. Immunoglobulin at the cell surface of hepatocytes was an inconsistent finding in all three groups and its presence was not related to areas of liver cell destruction. In studies using isolated hepatocytes, membrane-fixed IgG has been demonstrated in both HBsAg seropositive and seronegative CALD (Hopf et al., 1975). The absence of surface immunoglobulin staining does not completely rule out the possibility of an ADCC type reaction, as our method using tissue sections at light microscopical level might not be adequate for the study of this phenomenon.

Based on our results, in each of the three groups of CALD circumstantial evidence is present with sufficient consistency to allow for the construction of a pathogenetic immune mechanism. In IA-CALD, OKT8+ cells of both T cell and non-T, non-B cell origin, OKM+ lymphocyte-like cells, IgG producing plasma cells and polymorphonuclear leucocyte are prominent and constant features in areas with PMN. An ADCC type of liver cell destruction involving killer cells and IgG seems the main mechanism, but a contribution from OKM+ natural killer cells, and from OKT8+ cells in cell-mediated cytotoxicity can not be ruled out. In HB-CALD, OKT8+ cells of T cell origin are the only prominent and constant feature in areas with PMN, OKT8+ cells of non-T non-B origin and OKM+ cells being notably absent, while plasma cells were scanty. Cell-mediated cytotoxicity with OKT8+ cells of T cell origin thus seems to be the main immune mechanism. In PBC, prominent and constant features were mainly present in the central parts of portal tracts and included OKT4+ helper cells, and IgM producing plasma cells. In the areas with PMN, OKT8+ cytotoxic and plasma cells were constant features. The immune mechanisms in the portal tracts, known to include granuloma formation and correspondingly showing many T helper cells, seem to be mainly involving immunoglobulin production and thus a humoral immune reaction as also evidenced by literature data (Thomas et al., 1977). The localization of plasma cells surrounding bile ducts is highly suggestive in this respect. Cell mediated cytotoxicity with OKT8+ cells seems to be the main immune mechanism in areas with PMN.

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CHAPTER 3

Cellular and humoral immune reactions in chronic active liver disease. II. Lymphocyte subsets and viral antigens in liver biopsies of patients with acute and chronic hepatitis B*

H.F. Eggink, H.J. Houthoff, Sippie Huitema, G. Wolters, S. Poppema & C.H. Gips.

Departments of Pathology and Medicine, University Hospital and University of Groningen, Groningen and Organon Scientific Development Group, Oss, The Netherlands.

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Summary

The characteristics and distribution of the inflammatory infiltrate in liver biopsies of 25 patients with hepatitis B viral (HBV) infection were studied in relation to the distribution and expression of HBV antigens. Mononuclear subsets were characterized with monoclonal (OKT, OKM, Leu) antibodies to surface antigens. For the demonstration of viral antigens directly conjugated antibodies to surface (HBsAg), core (HBcAg) and 'e' (HBeAg) antigen were used. For the study of mutual relations all methods were performed on serial cut tissue sections. In chronic active hepatitis B (CAH-B, $n = 12$) OKT8+ lymphocytes of T cell origin were the only cell type present in areas with liver cell degeneration and T cell cytotoxicity appears to be the only immune mechanism. In chronic persistent hepatitis B (CPH-B, $n = 7$) the only conspicuous feature was the presence of many Leu 3+ lymphocytes of the helper/inducer population in the portal tracts. In acute hepatitis B (AHB, $n = 6$) OKT8+ cells of non-T origin (OKT1–, 3–) and Leu 7+ cells of presumed natural killer (NK) potential predominated in the areas with liver cell necrosis, and non-T cell cytotoxicity appears to be the predominant immune mechanism. In none of these disease entities a positive spatial relation could be established between the cytotoxic cells and the demonstrable expression of HBV antigens in hepatocytes. It is concluded that differences in immunological reaction pattern may explain the different course in the three forms of HBV infection studied.

Keywords chronic active liver disease hepatitis B lymphocyte subsets viral antigens

INTRODUCTION

The contribution of host defence mechanisms and viral antigenic expression in acute and chronic hepatitis B virus (HBV) infection of the liver are still incompletely understood.

As shown by the course of HBV infection during immunosuppression, the virus itself is not cytotoxic for hepatocytes and liver cell damage is related to the immunological reaction patterns of the host (Edgington & Chisari, 1975; Gudat *et al.*, 1975). The expression of HBV surface (HBsAg) or core (HBcAg) antigens on the cell surface or of other HBV-induced alterations of the liver cell membrane have been postulated as the main target antigens for host defence mechanisms (Edgington & Chisari, 1975; Ray *et al.*, 1976; Trevisan *et al.*, 1982). Based on *in vitro* studies, cell-mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC) are supposed to be the main immune mechanisms involved in the damage of virally infected liver cells, both in acute and chronic hepatitis B (Edgington & Chisari, 1979; Alberti, *et al.*, 1977; Thomas *et al.*, 1982).

Although the 'over-all' patterns of viral antigenic expression and the inflammatory infiltrate are mutually related (Gudat *et al.*, 1975; Ray *et al.*, 1976), an *in situ* cell-to-cell relationship between the hepatocytes with viral antigenic expression and the lymphocytes involved in CMC or ADCC has not been established. In a previous paper (Eggink *et al.*, 1982a) we have reported on the lymphocyte subsets in CAH, both HBsAg positive and negative, especially in relation to the areas with liver cell necrosis. The purpose of this study was to demonstrate a relation between the liver cells with detectable expression of HBV antigens (HBsAg, HBcAg, or the 'e' antigen: HBeAg) and the subsets of mononuclear cells possibly involved in the immunological attack with special reference to differences in acute hepatitis B (AHB), chronic persistant hepatitis B (CPH-B) and chronic active hepatitis B (CAH-B).

MATERIALS AND METHODS

Patient groups. During the period 1979-1982, from a total number of 36 patients with fully documented HBV infection and a liver biopsy, 25 cases were included in this study. According to clinical and histological parameters the cases fitted into the following groups: AHB ($n = 6$), CPH-B ($n = 7$) and CAH-B with or without cirrhosis ($n = 12$). Regular serum determinations of liver tests, HBV antigens, HBV antibody titres, immunoglobulin concentrations and antibody titres were performed in all cases of suspected or proven HBV infection according to protocol (Niermeijer & Gips, 1981). Some characteristics of the patient groups are given in Table 1.

Table 1. Clinical data of the investigated groups of patients with hepatitis B

	AHB	CPH-B	CAH-B*
No. of patients	6	7	12
Age (Years)	20-69	30-55	25-47
Female/male	2/4	2/5	2/10
Duration of illness (months)	<1	>6	>6
Serum			
HBsAg/anti HBs	6/-	7/-	12/-
anti-HBc IgM	6	7	12
HBeAg/anti-HBe	4/-	6/-	7/4†

AHB= acute hepatitis B; CPH-B = chronic persistent hepatitis B; CAH-B = chronic active hepatitis B.

* Including active cirrhosis.

† In one of the cases no HBeAg nor anti-HBe could be detected.

Between the groups with presence or absence of HBeAg in the serum and in the liver cell nuclei, no differences could be established in the activity of the inflammation nor in the relative contribution of the mononuclear subsets.

An additional group of five cases with characteristic distribution patterns of the HBV antigens in the liver parenchyma served as a reference covering the whole range of possible expressions of HBV antigens in hepatocytes. This group consisted of cases with early AHB, CPH-B with 'ground glass' hepatocytes, CPH-B with only HBcAg expression in the liver, CAH-B with HBsAg localization at cell membranes, and HBV infection in an orthotopic liver homograft with prominent expression of HBsAg en HBcAg at liver cell membranes.

The percutaneous liver biopsies were performed using a 1.6 mm Menghini needle. A small part of the biopsy was immediately frozen in freon-22 and stored at -70° C. The larger part of the biopsy was fixed in 4% paraformaldehyde with 5% glacial acetic acid and 6% $HgCl_2$, embedded in paraplast and used for light microscopy and some of the immunoperoxidase (IP) studies. For electron microscopy a very small part of the biopsy was fixed in phosphate-buffered 2% glutaraldehyde. Serial sections were cut

from all specimens to enable the study of relations between all parameters in each case. For each method sections of all cases were simultaneously incubated together with positive controls.

HBV antigens in liver sections. HBsAg, HBcAg and HBeAg were demonstrated using peroxidase (PO) conjugated anti-HBs, anti-HBc and anti-HBe immunoglobulins, respectively. These antibodies were kindly provided after testing by Organon Scientific Development Group, Oss, The Netherlands. For HBsAg 6 μm paraffin and frozen sections were used, for HBcAg and HbeAg 6 μm frozen sections were used. After pre-incubation with normal human AB serum diluted 1:5 for 15 min at room temperature the sections were incubated with diluted anti-HBs∞PO for 1 h at 37° C, and subsequently for the next 12 h at 4° C. Because the anti-HBe serum might show very weak anti-HBc reactivity, for the demonstration of HBeAg parallel sections were pre-incubated with normal human AB serum and subsequently with unconjugated anti-HBc serum for 1h at 37° C and the next 12 h at 4° C. The anti-HBc serum was free of anti-HBe. Thereafter one section was incubated with anti-HBe∞PO to demonstrate HBeAg, on the other section anti-HBc∞PO was applied to test the blocking activity of pre-incubation with anti-HBc. The sections were stained with diaminobenzidin (DAB) for 5 min.

Lymphocyte subsets in liver sections. The lymphocyte subsets were characterized with hybridoma produced, monoclonal antibodies (MoAbs) which were obtained from Ortho Pharmaceutical Corporation (Raritan, New Jersey, USA) and the Leu antibodies from Beckton and Dickinson (Mountain View, California, USA). The production, growth and characterization of these antibodies have been the subject of a series of recent reports (Reinherz *et al.,* 1980; Ledbetter *et al.,* 1981; Verbi *et al.,* 1982; Kay & Horwitz 1980; Breard *et al.*, 1980; Abo *et al.,* 1982).

To demonstrate reactivity of these lymphocyte subsets in tissue sections with MoAbs an indirect immunoperoxidase technique was used, as described in previous papers (Eggink *et al.,* 1982a, 1982b). In short, 6 μm thick frozen sections were air dried with ventilator for 30 min. The sections were fixed in acetone for 10 min at room temperature and shortly washed in PBS, pH 7.4 The sections were incubated with 25 μl of diluted antibody for 30 min. In addition, control sections were incubated with control ascitic fluid or PBS. All sections were subsequently incubated with peroxidase conjugated rabbit anti-mouse Ig serum (Dakopatts, Copenhagen, Denmark) diluted 1:20 for 15 min, supplemented wit 1% human AB serum. Between incubations the

sections were washed for 10 min in three changes of PBS. The sections were stained with 3-amino-9-ethylcarbazole and H_2O_2 during 10 min. Nuclear counterstaining was performed with Haemalaun and the sections were mounted with Aquamount (Gurr, Essex, UK).

Immunoglobulins and plasma cells in liver sections. Plasma cells and immunoglobulins at the surface of hepatocytes were demonstrated in 4 μm thick paraffin sections. After pre-incubation with normal rabbit serum diluted 1:15 at room temperature for 10 min, sections were incubated with peroxidase conjugated rabbit anti-human IgG, IgM or IgA serum (Dakopatts) for 60 min at 37° C. These sections were stained with DAB for 5 min and counterstained with Haemalaun. For every technique, control sections were incubated with normal serum or PBS in the first step.

Serological methods; additional to general protocol. The determinations of HBV antigens and antibodies in the serum of all patients in this study, taken at the time of liver biopsy, were repeated. HBsAg and anti-HBs were determined by solid phase radioimmunoassay (Abbot Diagnostic Division, North Chicago, USA), HBeAg and anti-HBe by enzyme immunoassay, anti-HBc total and anti-HBc-IgM, the latter as a marker of recent or ongoing HBV infection (Niermeijer *et al.,* 1978; Aldershvile *et al.,* 1981) were determined by enzyme immunoassay (Organon, Oss, The Netherlands).

RESULTS

The histology of the liver biopsies in each of the groups was fully in accordance with the diagnostic criteria for AHB, CPH-B en CAH-B, respectively (Bianchi *et al.,* 1977). In the reference group, the results with peroxidase conjugated antibodies to HBsAg, HBcAg and HBeAg were similar to those with unconjugated antibodies in an IF technique (Houthoff *et al.,* 1980). Furthermore, the presence and distribution of surface and core particles in EM closely correlated with the results of the IP and IF methods (Fig. 1).

In general, hepatocytes and portal tract structures were negative with all methods, apart from some immunoglobulin staining of the portal vessels and mesenchyme. The endothelial and Kupffer cells were negative with all methods, except for the Leu 3 antibody that showed weak staining of Kupffer cells. No OKT6+ lymphocytes were found in any of the cases. The distribution in the liver of the mononuclear cell subsets (Fig. 2), characterized with the MoAbs, is summarized in Table 2.

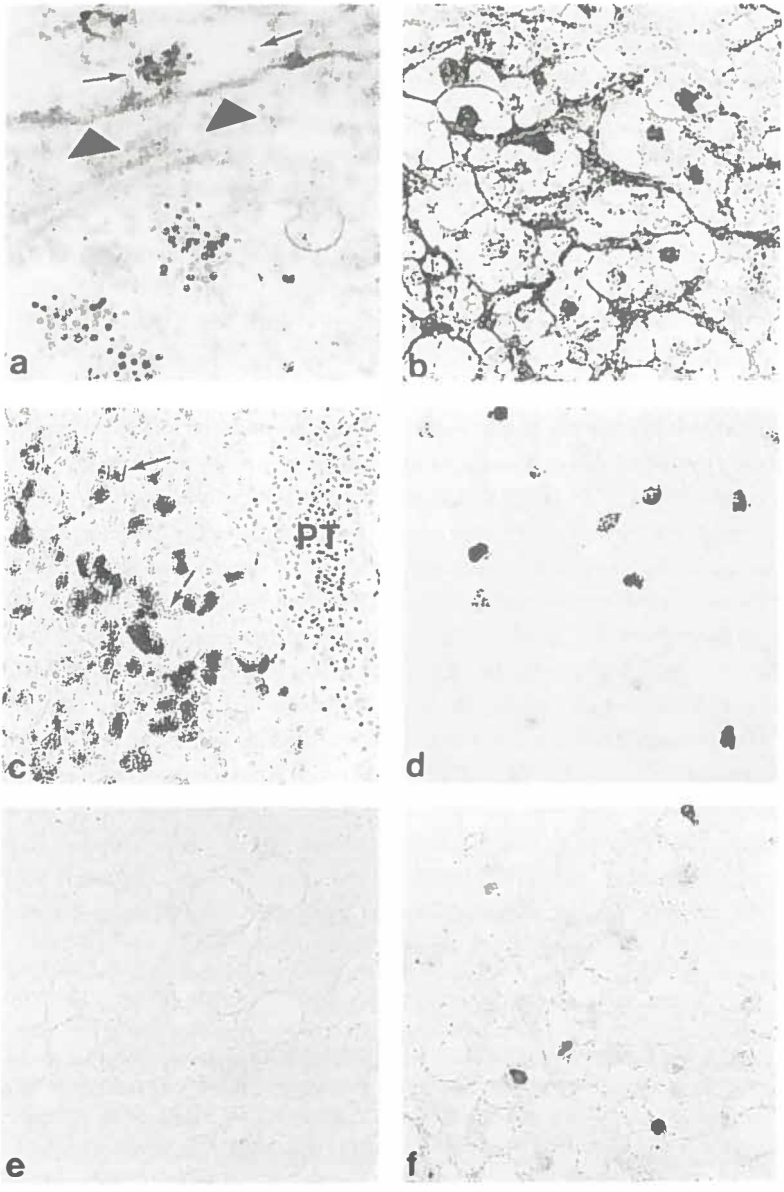
Table 2. Distribution of the mononuclear cell subsets in liver biopsies of patients with AHB, CPH-B and CAH-B

Subsets	Functional interpretation	Liver parenchyma			Portal tracts		
		AHB	CPH-B	CAH-B	AHB	CPH-B	CAH-B
Leu 3+	inducer/helper T cell	±	–	–	–	++	+
OKT8+, 11+, 1+, 3+	cytot/supp T cell	±	±	++	+	+	+
OKT8+,11+, 1 ,3-	(?) cytot non-T cell	++	–*	–	–	–	–
Leu 7+	NK cell	+	–	–	±	–	–
OKM1+,2+	K/NK cell	–	±	±	–	–	–
IgM+	B cell	±	–	–	+	–*	–

– = absent, ± = some, + = appreciable number, ++ = many.
* Except for one case of otherwise proven CPH-B.

In all cases of AHB some IgG positive plasma cells were present, mainly in the portal tracts. Immunoglobulins at the surface of hepatocytes were present in three cases, randomly distributed throughout the parenchyma. The main characteristics in AHB were the absence of demonstrable HBV antigens and the preponderance of non-T cells in the inflammatory infiltrate. In CAH-B plasma cells with IgG and IgA were scanty in most cases, plasma cells with IgM were absent.
Immunglobulins at liver cell membranes were present in five cases, mostly randomly distributed throughout the parenchyma and at not especially

Fig. 1. Hepatitis B viral particles and the immunohistologic detection of viral antigens in the liver parenchyma. (a) Hepatitis B core particles (small arrows) in the cytoplasm of a hepatocyte near the plasma membrane (arrowheads) and in the adjacent extracellular space. Electron micrograph, magnification x 48,450. (b) Expression of HBcAg in the nuclei and plasma membranes of liver cells; to a lesser extent HBcAg is also present in the cytoplasm. anti-HBc ωPO, magnification x 350. (c) Expression of HBsAg in the cytoplasm of many hepatocytes. Also, some HBsAg is present in relation to liver cell membranes (arrows). In a portal tract (PT) an inflammatory infiltrate is present with some spillover on the periportal parenchyma. Anti-HBs ωPO with light haematoxylin counterstaining, magnification x 140. (d), (e) and (f) are from the same case and illustrate the differential detection of HBcAg and HBeAg, magnification x 350. (d) Incubation with anti-HBCωPO, showing HBcAg in liver cell nuclei. (e) Same, but pre-incubation with unconjugated anti-HBc. The HBcAg in the nuclei has been completely blocked. (f) Pre-incubation with unconjugated anti-HBc, incubation with anti-HBeωPO, showing HBeAg in liver cell nuclei.


a
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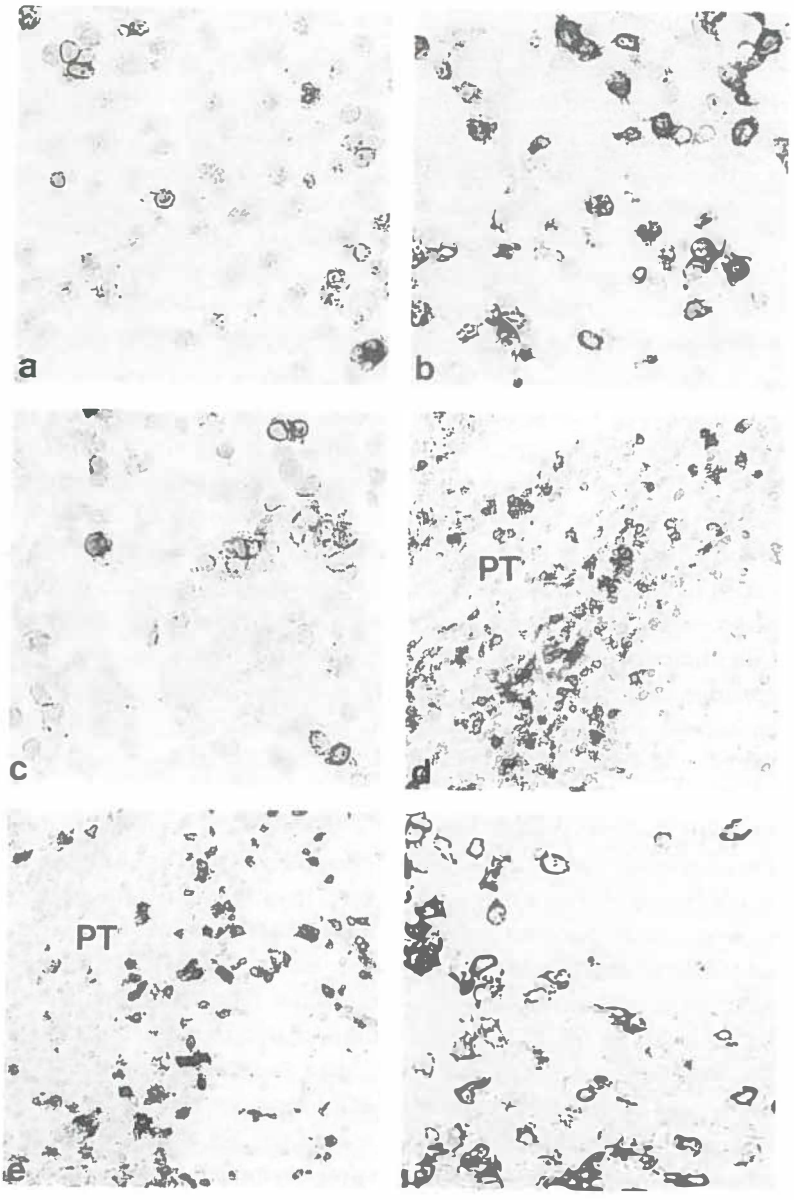
related to areas with PMN. At least one of the HBV antigens was present in hepatocytes in every case. In seven cases hepatocytes with a cytoplasmic HBsAg localization were found, their distribution and amount varied greatly from patient to patient, but also from area to area. Intranuclear HBcAg was found in randomly distributed hepatocytes of eight cases, in one case in combination with cytoplasmic HBsAg. HBeAg was present in seven cases. It was consistently and only found in the nuclei of hepatocytes, that also showed HBcAg, all the patients with nuclear HBeAg were also HBeAg seropositive. A liver cell membrane related localization of HBcAg, HBsAg or HBeAg was not found. The positive reaction with anti-HBc and anti-HBe was totally blocked by pre-incubation of the liver sectins with unconjugated antisera of analogous titre and specificity, but it remained unaffected by pre-incubation with normal human sera. In the inflammatory infiltrate the main characteristic in CAH-B was the nearly exclusive presence of OKT8+T cells in parenchyma. In CPH-B immunoglobulins at the surface of the hepatocytes were found in three cases. Cytoplasmic HBsAg was present in all cases, in two cases also a membrane related localization was found. Of the eight cases with demonstrable HBcAg, in four cases nearly all hepatocytic nuclei were positive, in one case only some nuclei were positive, while in two cases nuclear, cytoplasmic and membrane related HBcAg was found. HBeAg was present in hepatocytic nuclei of six cases. The inflammatory infiltrate in CPH-B remained confined to the portal tracts, its main feature was the large proportion of Leu 3+ T cells. A definite spatial relationship between liver cell necrosis and/or lymphocytic infiltration and/or a lymphocyte subset on one hand and the presence of on of the HBV antigens on the other was not found in any of the cases. In CAH-B the HBV antigens were less frequently found in the periportal parenchyma than in the other parenchymal zones. A negative correlation could not be established, however.

Fig. 2. Immunohistological demonstration of the phenotype of lymphocyte subsets in the liver. (a-c) Liver parenchyma in AHB (a) Leu 7+, (b) OKT8+ cells and (c) OKT1+ cells. Note the relatively small number of pan T phenotypic cells in (c) as compared to the cells of suppressor/cytotoxic phenotype in (b) magnification x 350. (d & e) Consecutive sections of the same case, chronic persistent hepatitis B with some spillover of lymphocytes from the portal tract (PT) on the adjacent parenchyma. OKT1+ cells (d) are mainly present in the portal tracts, whereas OKT8+ cells (e) are mainly found in the periphery of the portal tracts and in the periportal parenchyma, magnification x 140. (f) CAH-B. OKT8+ cells in a periportal area with piecemeal necrosis, magnification x 350.


a
b
c
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PT

DISCUSSION

Uncomplicated, self limited AHB, CPH-B and CAH-B with or without cirrhosis represent three syndromes in HBV infection. The morphology and serology of CPH-B and CAH-B have been shown to occur transiently during the early course of typical self limited AHB (Houthoff *et al.,* 1980); the chronic conditions may thus be interpreted as the persistence of early stages of infection. The virus itself is not cytotoxic for liver cells and the differences between the three syndromes are supposed to depend at least in part upon different patterns of host's immune response. As shown in Table 2, there are indeed differences in the lymphocyte subsets between the three syndromes, both in the liver parencyma and to lesser extent in the portal tracts. The expression of HBV antigens on the liver cell surface has been postulated as the main target for the immunological reaction (Edgington & Chisari, 1975). In other viral hepatitis forms, e.g. in cytomegalovirus infection, a periportal or CAH type of hepatitis correlated with the presence of viral antigens in the periportal hepatocytes (Ten Napel, Houthoff & The, 1983). In a similar way, we tried to relate the mononuclear cell reaction to the presence of HBsAg, HBcAg and/or HBeAg in hepatocytes. If antigenic expression participated in the immune response, there should at least be a positive spatial correlation between the lymphocytic effector cells and the hepatocytes with demonstrable HBV antigens, this could not be demonstrated, however.

Membrane fixed immunoglobulins were an inconsistent finding, not related to areas with liver cell destruction, and although in agreement with the findings in other studies (Hopf *et al.,* 1975) do not seem to be involved in the pathogenesis either. In the serum, the presence of HBeAg or anti-HBe was not related to the mononuclear subsets involved, nor to the severity of the clinical or histological symptoms. For anti-HBe, this is in contrast to other reports (Eleftheriou *et al.,* 1975), but in general the serological findings are in accordance with other studies (Aldershvile *et al.,* 1981; Niermeijer *et al.,* 1978). An explanation of our findings might be that hepatocytes with viral replication more than those with expression of viral antigens are involved in the inflammatory response (Burrell *et al.,* 1982) and/or that other liver cell membrane antigens are also involved in the cytotoxicity reaction. *In vitro* studies with peripheral blood lymphocytes from patients with chronic HBV infection have shown cellular immunity to heterologous target cells coated with HBsAg (Warnatz *et al., 1979*). Based on the presence of the E rosette receptor it was concluded that these cells were T lymphocytes. However, it has been shown that the E rosette receptor

is present both on T lymphocytes and on a population of non-T cells with NK or K activity (Verbi, *et al.*, 1982). It also became clear that T lymphocytes as effector cells not only possess a specificity for viral antigens, but also require an additional recognition of HLA-A, B, C system (Dickmeiss, Soeberg & Sweygaard, 1977). Based on this data the effector cells in several *in vitro* studies were probable not T lymphocytes but NK or K cells. With MoAbs it is possible to define different subsets of T and non-T cells, both in peripheral blood and in the tissues. In a previous study (Eggink *et al.*, 1982a) we reported that during CAH-B OKT8+ cells of T cell origin (OKT1+, 3+, 11+) were the predominant mononuclear cell type in the parenchyma of the liver and that these T cells were related to areas with liver cell necrosis. The results in this study corrobate this data. The HLA-A, B, C antigens are normally poorly expressed on hepatocytes but their expression is enhanced during viral infection and reactive conditions (Thomas *et al.*, 1982). As in the periportal areas of CAH-B these reactive circumstances are present and the presumably cytotoxic T cells are the only cell type found, it thus seems justified to conclude that T cell cytotoxicity under these circumstances is the only possible immune mechanism involved in the 'Piecemeal' necrosis of hepatocytes.

In CPH-B the main and characteristic features of the inflammatory infiltrate were the abundance of Leu 3+ helper/inducer cells in the portal tracts and some mononuclear cells in the liver parenchyma. Immunological tolerance of the viral antigens or low expression of HLA antigens may be involved in CPH-B.

In AHB mononuclear subsets in the parenchyma were essentially of non-T, non-B cell origin, although some OKT8+ T cells were also precent. The two mainly occuring subsets were a population of NK cells (Leu 7+, Abo *et al.*, 1982) and a population of OKT8+ non-T cells (OKT1–, 8+, 11+, Eggink *et al.*, 1982).

So far, the latter population has not been characterized functionally with *in vitro* methods, but its presence in areas of liver cell necrosis, both in AHB and in hepatitis of idiopathic autoimmune type, suggests a specific function in these syndromes. One of the possibilities might be that these OKT8+ non-T cells are the effector cells in a non-T cell cytotoxic reaction, either as K cells in ADCC or as NK cells in CMC. The finding of Leu 7+ cells in AHB, is in agreement with the known occurrence of NK cells in other instances of acute immune reactions (Herberman *et al.*, 1982). The complete absence of OKM+ cells in AHB is noteworthy in this context. Although Leu 7+, OKT1–,

3–, 8+, 11+ and OKM+ all specify populations of non-T mononuclear cells with proven or presumed NK/K activity, these membrane antigens are not present on the same cell and appear to specify functionally different subsets.

In conclusion, our results demonstrate that (1) both T cells (OKT1+, 3+, 8+, 11+) and non-T cells (OKT1–, 8+, 11+, Leu 7+) may be involved in a cytotoxicity reaction during hepatitis B infection; (2) the membrane markers for mononuclear non-T cells with potential or proved K/NK activity (OKT1–, 3–, 8+, 11+, Leu 7+, OKM1+, 2+) specify different subsets; (3) the expression of Hbs/c/e Ag seems not to be directly involved in the immune reaction; (4) different immune mechanisms occur in AHB, CPH-B and CAH-B, with probably NK cell cytotoxicity in AHB and T cell cytotoxicity in CAH-B and (5) a defect in the activity of a population of NK cells (Leu 7+) may be a major factor in the occurrence of CAH-B.

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CHAPTER 3 ADDENDUM

T-Cell Subsets in Liver Diseases

H.F. Eggink MD, H.J. Houthoff MD, PhD & S. Poppema MD.

Department of Pathology, University of Groningen, Groningen, The Netherlands

In a recent issue, Pape *et al.* (1) reported on cytotoxic suppressor T cells in the liver of patients with various liver diseases. They state that little is known about the occurrence and distribution of cytolytic lymphocytes in the liver. Unfortunately, in their literature review, they may have failed to find the publications of us (2,3) and others (4) that not only deal with similar topics, but also report on the same disease entities. Nor do the authors comment on the abstracts (5,6) that dealt with the same problems and were presented during the same meetings as the abstract on part of their own study (7). Pape *et al.* conclude that cytotoxic T-cells are the main cell type involved in lymphocyte-mediated cytolytic mechanisms in these conditions and thus constitute a common final pathway. As this conclusion is at variance with ours, we take the opportunity to challenge their viewpoints.

Working with monoclonal antibodies from the OKT and Leu series, our results with antibodies defining the phenotype of pan T cells, T cytotoxic/suppressor cells, and NK cells were similar to those of Pape *et al.*, in acute viral infection (5,8), chronic hepatitis B (2,8), and primary biliary cirrhosis (2). However, the differences in interpretation are mainly due to the methodology of infiltrate analysis. *In the first place,* the phenotype of a given lymphocyte subset may rather be judged by the application of a panel of monoclonal antibodies than by a limited selection; the former results in completely defined subsets, for example OKT1–3–4–8+11+ Leu 7– and OKT1–3–4–8–11– Leu 7+ are two distinct subsets of non-T, non B lymphocytes. It has not only been shown that OKT8 (or T811) define both T and non-T lymphocyte subsets (1,2,8,9), but also that distinct subsets of non-T non-B lymphocytes (OKT1–3–. T411–) exist of which only some express OKT8 (or T811). The absence of HNK1+ (= Leu 7+) cells is thus no proof for the assumption that K/NK cells are lacking in a T811+ infiltrate, as the authors suggest. *In the second place,* only by the characterization of all the lymphocyte subsets in an inflammatory infiltrate, and thus again in using a panel of monoclonals, the differences and similarities between the infiltrates

in various disease entities may hint at the main pathogenetic mechanisms. Thus, Pape *et al.* showed that in chronic hepatitis B 34% of the cells were of T cytotoxic/suppressor phenotype (74% of the 47% T cells) and in PBC 36.5% (58% of the 63% T cells). Although the percentage of total percentage of T cells is significantly higher. Indeed, we found that T inducer/helper cells (Leu 3+) were the predominant subset in PBC, especially prominent in the central parts of portal tracts. The combination of many T helper cells with B cells and plasma cells is characteristic for the inflammatory infiltrate in the portal tracts of PBC and points toward a possible humoral component in the immune reaction. *In the third place,* the assumption that the cytolytic T cells are attacking infected hepatocytes, as the authors suggest, is only valid if the markers of infection in the liver parenchyma coincide with the distribution of the inflammatory infiltrate. Although this may be true in acute viral hepatitis, it is not the case in chronic hepatitis B (infection throughout the parenchyma and portal/periportal inflammation) (8) and obviously not in PBC either.

In conclusion, it appears that by differences in methodological approach the otherwise similar results in Pape's and our studies have lead to different interpretations. Based on our results, K/NK cells are an important defence mechanism in acute viral hepatitis; not an uncontrolled effect of cytolytic T cells, as Pape *et al.* conclude, but a deficient K/NK cell activity in the start may contribute to the progression toward chronic hepatitis B, and in PBC a possible humoral component plays an important role in the immune attack. Therefore, a common final pathway in these and other immune-mediated liver diseases is unproven and to our opinion also unlikely, although T811+ cells do occur in all these conditions. As the understanding of pathogenetic mechanisms in liver diseases is fundamental for the design and interpretation of new therapeutic trials, we feel that the final solution of these problems will be of practical value in clinical hepatology.

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CHAPTER 4

Histopathology of Serial Graft Biopsies From Liver Transplant Recipients

H.F. Eggink MD, N. Hofstee MD, C.H. Gips MD PhD, R.A.F. Krom MD & H.J. Houthoff MD PhD.

From the Departments of Pathology, Medicine, and Surgery, University Hospital and University of Groningen, Groningen, The Netherlands.

Summary

Serial graft biopsies ($n = 78$) from 12 liver transplant recipients (followed clinically up to 47 months) were studied with the use of histology, histochemistry, immunostaining, and electron microscopy. Planned-protocol needle biopsy specimens were taken from the graft before removal from the donor, 1 hour after transplantation, on the eighth day, and at yearly intervals. Nonprotocol biopsies were taken when deterioration of the clinical condition made a decision on changes in the regimen necessary. The *protocol biopsies* provided a baseline for graft condition and diagnostic histopathologic features. In these biopsies signs of hyperacute rejection, chronic rejection, or the recipient's previous liver disease were not observed. Mild acute rejection was regularly present on the eighth day, possibly due to a lag phase in the effect of immunosuppression. The syndromes in the *nonprotocol biopsies* included 'pure' parenchymal cholestasis, reversible acute rejection resembling chronic active hepatitis, viral infection, and acute bacterial cholangitis. Each of these syndromes correlated with a separate histopathologic entity. Therefore, these entities proved to be of diagnostic value. It is concluded that a graft biopsy may substantially add to the pathogenetic interpretation, differential diagnosis, and management of major graft syndromes in orthotopic liver transplant recipients.

INTRODUCTION

Orthotopic liver transplantation (OLT) has been performed worldwide in well over 550 patients and has proved to be of value as the ultimate treatment in various forms of irreversible liver disease. Most reports on liver graft pathology deal with autopsy findings[1-4]; pathologic data are still rather scanty in comparison to the literature on graft pathology in other organs such as, for example, the kidney or the skin,[5] however. Serial graft biopsies from liver transplant recipients usually have not been included in the screening and follow-up of impaired graft functioning, although incidentally graft biopsies have been performed for the evaluation of major graft syndromes.[2]

When OLT started to be performed in the University Hospital of

Groningen, the transplantation team decided that liver biopsy pathology should document and contribute to the clinical diagnosis of the major graft syndromes.[6 7] For the pathologic interpretation of the graft biopsies, the morphology in protocol biopsy specimens taken before removal from the donor, 1 hour after OLT, on the eighth day, and at yearly intervals served as a reference.[8] Thus, the fluctuations in liver disease and their relation to the functional and serologic follow-up could be studied longitudinally in a series of graft biopsies per patient. The aim of this study was to evaluate the diagnostic contribution of graft biopsy interpretation in the recognition and separation of histopathologic entities related to the major graft syndromes.

At the start, no conclusive data could be found in the literature to differentiate between the histopathology of the various graft syndromes, at least not to the extent that the histopathologic diagnosis could reliably be used as a basis for far-reaching changes in the therapeutic regiment. Thus, in comparing the histopathologic features with clinical and serologic parameters and the reaction to changes in the immunosuppressive regimen, circumscribed pathologic features were tentatively linked to clinical syndromes and subsequently used to test this relation in analogous situations in the next patients. In giving an overview of the interpretation and differential diagnosis of the pathologic findings in liver graft biopsies, we demonstrate the relevance of these biopsies in the clinical management of patients with OLT.

GENERAL TRANSPLANTATION DATA

OLT was made possible by the formation and cooperation of the University Liver Transplant Team, to cover the transplantation problems in medicine, surgery, anesthesia, histocompatibility testing, microbiology, radiology, and pathology. Selection criteria for possible recipients, pretreatment, surgery and follow-up were handled according to a protocol.[6 7] Donor livers were from hitherto healthy patients meeting the criteria of cerebral death in the absence of sepsis. The surgical technique was modified from the methods described by others.[9 10] The cholecystojejunostomy and 'gall bladder conduct' were replaced by an end-to-end choledochus anastomosis,[11] and special care was taken to provide an optimal blood supply to the bile ducts. For prevention of infection with gram-negative microorganisms, a selective enteric decontamination was performed preoperatively.[12]

The postoperative immunosuppressive regimen consisted of prednisolone/azathioprine with initially daily doses of 200 mg and 2 mg/kg, respectively, and a subsequent decrease of the prednisolone dosage. In cases of acute graft rejection the immunosuppressive treatment was temporarily increased. Graft function was followed by biochemical and serologic parameters according to a protocol. In all graft recipients, two peaks of serum aminotransferases occurs: directly after OLT and from the sixth till the tenth postoperative day. Planned protocol needle biopsy specimens were taken from the graft before removal from the donor, 1 hour after OLT, on the eighth day, and at yearly intervals. Further graft biopsy specimens were taken when deterioration of liver function made a decision on changes in the clinical regimen necessary.

PATIENT DATA

Data on the first 12 OLT recipients with a follow-up period of 4 years (March 1979 to March 1983) is included in this study. The clinical course of most patients has been described elsewhere.[6 13] Some data relevant to this study is summarized in Table 1. As shown in this Table, 7 patients are in excellent condition without signs of clinical morbidity. The other 5 patients died within 4 months after OLT; in all these cases a complete autopsy has been performed. The preoperative condition of the patients who died was bad in 3 cases; this was most consistently reflected by severe coagulation disorders. In total, 78 graft biopsies were studied, 34 of which were nonprotocol.

MATERIALS AND METHODS

Percutaneous graft biopsies were performed with a Menghini needle 1.6 mm in diameter. A long biopsy specimen (4 cm) was taken, from which parts were used for microbiologic purposes, routine histologic and immunoperoxidase (IP) studies, and electron microscopy (EM); and a part was immediately frozen for histochemical and immunofluorescence (IF) purposes.

For histologic study, the tissue was fixed in 4% formaldehyde with 6 g/dl $HgCl_2$ and 5% glacial aceticacid for 1 hour at room temperature, dehydrated, and embedded in paraplast. Staining methods on sections 4 μ in thickness included hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) following diastase digestion, Gomori's reticulin, azan, and Perls' iron stain.

Table 1. Patient Data

OLT no.	Age (years)	Sex	Clinical diagnosis	Preoperative condition: Coagulation disorders	Ascites	Encephalopathy	Postoperative condition: Induced rejection	Viral infection*	Bacterial infection*	Cause of death†	Survival (months)	Graft biopsies: Protocol	Nonprotocol
1++	20	F	IAH	++	–	+	–	–	–	CDH	0	–	–
2	44	F	PBC	±	+	+	+	CMV	–	–	47	4	9
3	42	F	HCC	–	–	–	+	HSV	–	–	41	6	5
4	55	F	CC	++	–	++	–	–	ser.	CDH	1	3	1
5	53	F	IAH	+	+	–	+	HSV	–	–	37	6	3
6	54	F	IAH	++	+	–	–	–	–	CDH	0.5	3	–
7	54	M	CC	–	–	–	+	HSV	–	–	29	5	4
8	50	F	PBC	–	–	–	+	CMV	–	–	26	4	4
9	46	F	PBC	±	–	–	–	–	ent.	CDH	1.5	3	2
10	58	F	PBC	–	–	–	–	–	–	–	21	4	3
11	49	F	PBC	–	–	–	–	CMV	–	–	18	4	3
12	47	F	PBC	–	–	–	–	–	–	HAT	0.25	2	–

CC, crytogenic cirrhosis; CDH, coagulation disorders with hemorraghes and hematomas; CMV, cytomegalovirus, ent., enterococcus; HAT, hepatic artery thrombosis; HCC, hepatocellular carcinoma; HSV, herpes simplex virus; IAH, idiopathic autoimmune hepatitis; OLT, orthotopic liver transplantation; PBC, primary biliary cirrhosis; ser., serratia.

* Infections verified by increased antibody titers, isolation, and/or culture. Monitoring for viral infection included tests for herpes simplex virus, cytomegalovirus, hepatitis B virus (HBV) and hepatitis non-A, non-B virus (NANB). Serologic parameters for HVB and NANB were always negative.

† At autopsy no evidence of graft rejection, sepsis, bacterial cholangitis, or any pathology of the surgical anastomoses was found.

++ Because no graft biopsies were obtained from OLT 1, no further mention of this case will be made in the text.

For histochemical study, the tissue was snap-frozen in freon-22. Enzyme histochemical methods used on cryostat sections 6 μ in thickness included those for the demonstration of NADH tetrazolium reductase, acid phosphatase (diazo method), alkaline phosphatase, Mg^{2+} dependent adenosine triphosphatase (ATPase), and leucine aminopeptidase.[14]

EM STUDIES

For EM studies, the tissue was cut in small parts, fixed in phosphate-buffered 2% glutaraldehyde for 4 hours, postfixed in phosphate-buffered 1% OsO_4 for 1 hour, and after dehydration embedded in Epon. Sections 1 μ in thickness were stained with 0.5% toluidine blue and used for light-microscopic study. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

IF/IP STUDIES

Cytomegalovirus (CMV) antigens, including early (CMV-EA) and late (CMV-LA) antigens, were demonstrated with the use of IF and IP methods on frozen and paraffin-embedded sections, according to methods described in previous papers.[15] [16] In short, 6-μ paraffin sections were incubated with diluted human anti-CMV-EA/LA gamma globulin for 1 hour at 37 C with continued incubation at 4 C overnight. The gamma globulin was directly conjugated to FITC or to horseradish peroxidase (HRP). For the peroxidase reaction, the slides were stained with the diaminobenzidin (DAB) reaction for 5 minutes. Between the steps the sections were thoroughly rinsed in phosphate-buffered saline (PBS).

Herpes simplex virus (HSV) antigens were demonstrated with the use of diluted rabbit anti-HSV serum (DAKO, Copenhagen, Denmark). In a second step, goat anti-rabbit Ig conjugated to FITC or to HRP was used.

Hepatitis B virus antigens, including surface (HBsAg), core (HBcAg), and e (HBeAg) antigen, were demonstrated with the use of peroxidase-conjugated antibodies (Organon, Oss, The Netherlands) as previously described.[17]

Fibrin-related products (FRPs), included fibrinogen, fibrin monomers (FM), and fibrin degradation products (FDP). These were demonstrated on 6-μ frozen sections as described previously.[18] [19] In short, in the first step a

diluted antibody against FM, fibrin, or FDP was applied, followed in the second step by FITC-conjugated goat-anti-rabbit Ig.

Routinely, in each incubation, liver tissue, with and without these antigens, served as a positive or negative control, respectively.

RESULTS

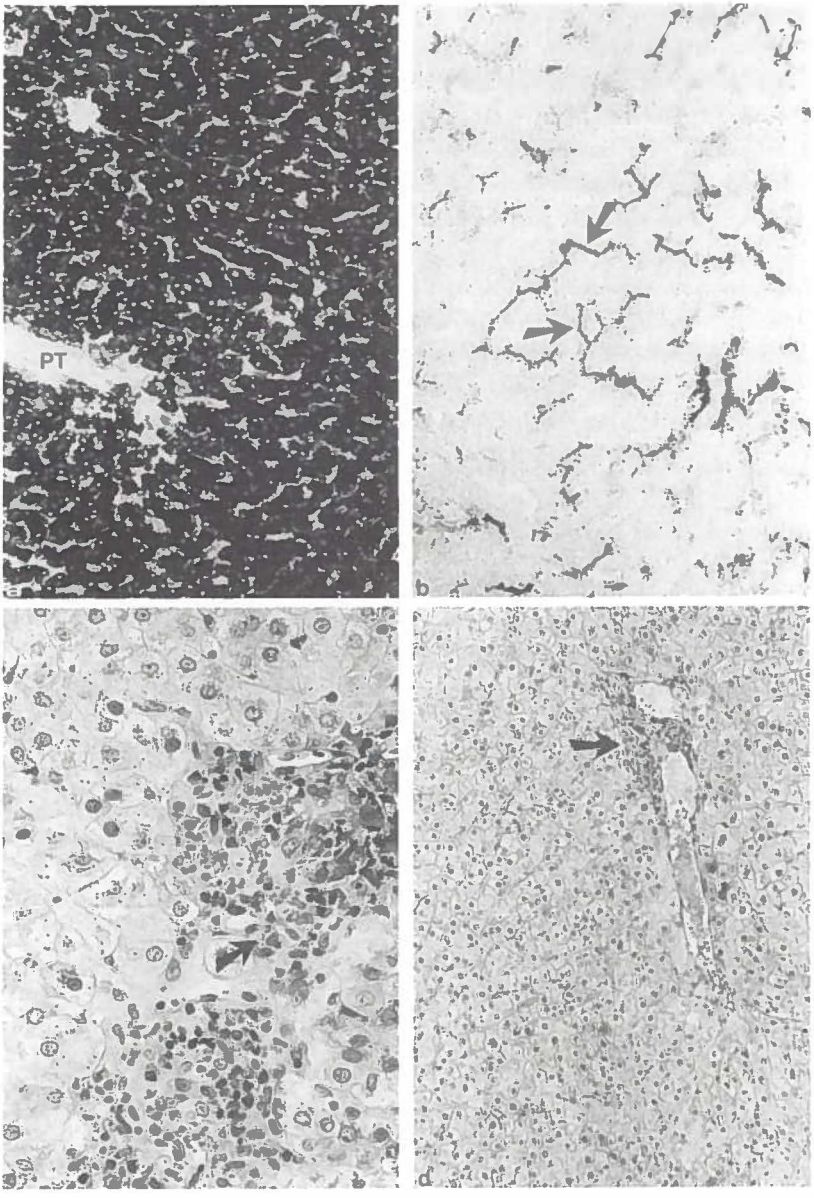
HISTOPATHOLOGY

Protocol Biopsies (Figure 1a-d)

In the first two protocol biopsies from each graft, before preservation and 1 hour after OLT, pathologic changes were nearly always absent. Sometimes some scattered granulocytes in the parenchyma were observed in the second biopsy in cases with long duration of transplantation. Liver cell necrosis, intravascular coagulation, or signs of hyperacute rejection were never observed. In the graft for OLT 3, fibrosis and inflammatory infiltrates with granulocytes, lymphocytes, and plasma cells were present in portal areas, together with granulocytic infiltration of bile duct epithelium, lympho-histiocytic aggregates in the parenchyma, and moderate periportal cholestasis.

In the third protocol biopsy, taken on the eighth postoperative day, identical pathologic changes were present in 6 cases. These consisted of slightly enlarged portal tracts and portal inflammatory infiltrates with lymphocytes, granulocytes, and plasma cells. Some spillover of lymphocytes onto the adjacent periportal parenchyma was occasionally noted, but piecemeal necrosis of periportal hepatocytes and bridging or confluent hepatic necrosis of parenchymal areas were always absent. Regularly, in the third acinar zone, slight to moderate cholestasis was observed. Invasion of bile duct epithelium by some lymphocytes was present in 5 cases. Both the

Figure 1. Protocol liver graft biopsies. (a) One hour after OLT. A portal tract (PT) is negative, the hepatocytes show a normal activity without early signs of irreversible degeneration (NADH tetrazolium reductase reaction, x 140). (b) One hour after OLT. Normal polarity of the hepatocytes with the enzyme activity confined to the bile canalicular area (arrows). (Leucine aminopeptidase, x 350). (c) Eight days after OLT. Lymphocytes and some plasma cells in a portal tract, interpreted as mild acute rejection. Invasion of bile duct epithelium *(arrow)* or spillover on the adjacent liver parenchyma is absent (H&E, x 350). (d) Two years after OLT. Note the slightly enlarged portal tract *(arrow)*, the absence of portal inflammatory infiltrate and the normal parenchyma. Interpreted as a morphologic normal graft. (H&E, x 140).


PT
a
b

disturbed liver function and the biopsy pathology proved to be reversible without changes in the clinical regimen. In 2 cases (OLT 6 and 10) coagulative necrosis was found in the third protocol biopsy; the necrosis was not accompanied by any inflammatory infiltrate. In 1 case (OLT 11) only nonspecific reactive changes were present. The biopsies after yearly intervals only showed some portal fibrosis and a minimal lymphocytic infiltrate. Plasma cells and spillover of lymphocytes were absent.

Nonprotocol Biopsies (Figure 2)

Histopathologic features of the nonprotocol biopsies are grouped according to the main features (Table 2). A first entity consisted of changes

Table 2. Histopathologic Characteristics and Interpretation of the Main Graft Syndromes

Histopathologic characteristics	Interpretation
CPH with portal plasma cells (third protocol biopsy)	Acute rejection, mild
CALD with PMN and mild parenchymal cholestasis	Acute rejection
-id- with BHN or confluent necrosis	Acute rejection, severe
Random AD of individual hepatocytes, minimal inflammation	Viral infection during IS
Acute or chronic cholangitis with parenchymal cholestasis	Bacterial cholangitis or bile duct pathology
Pure parenchymal cholestasis	Functional graft insufficiency

CALD, chronic active liver disease; PMN, piecemeal necrosis; BHN, bridging hepatic necrosis; AD, acidophilic degeneration; IS, immunosuppression; Dd differential diagnosis; CPH, chronic persistent hepatitis.

closely resembling chronic active liver disease (CALD, $n = 9$) (Figure 3). Plasma cells, lymphocytes, and granulocytes in the portal and periportal areas, piecemeal necrosis, and some lymphocytic infiltrate in the other parenchymal areas constituted the main and constant features. Bridging or confluent hepatic necrosis were sometimes present, and mild cholestasis in the third acinar zone was a common feature. Occasionally a lymphocyte was

present in bile duct epithelium, but the features of acute or chronic cholangitis were never conspicuous. Clinically, this entity correlated with episodes of increased graft dysfunction and fever. It always occurred during periods of low daily doses of immunosuppressive therapy, and on two occasions (Biopsies 2.7 and 3.4) during forced and total withdrawal of immunosuppression for at least 5 days on behalf of a previous episode of viral infection. The fever during these episodes always proved to be rapidly reversible by a moderate increase of the daily dose of prednisolone.

A second entity ($n = 8$, figure 2b-d) consisted of spotty necrosis with mainly acidophilic and sometimes ballooning degeneration of individual liver cells without appreciable parenchymal inflammation. Occasionally, some lymphocytic infiltrate in the portal tracts was found. No spillover of lymphocytes onto the adjacent parenchyma, piecemeal necrosis, or invasion of bile duct epithelium was present. This entity preceded and/or accompanied clinical episodes of viral infection, as evidenced by a rise in serum antibody titers or positive cultures.

A third entity ($n = 4$, Figure 4e) consisted of portal inflammatory infiltrates and appreciable parenchymal cholestasis. The inflammatory infiltrates were located mainly around bile ducts, and infiltration of bile duct epithelium was always present. The infiltrates consisted mainly of granulocytes, with some scattered lymphocytes and plasma cells. This entity correlated with episodes of jaundice and/or bacterial infection; in 1 case *Staphylococcus epidermidis* was cultured from bile.

A fourth entity ($n = 3$, Figure 4a-d) showed pure parenchymal cholestasis as the only conspicuous feature. The cholestasis varied from moderate to massive; no inflammatory cells were present in the parenchyma or in the portal tracts. Pure cholestasis mainly occurred during coagulation and circulation disorders; it was the main feature in the graft biopsies from the patients with preoperative severe coagulation disorders.

SPECIAL METHODS

IF and IP Methods

In none of the biopsies could CMV-LA, HSV, HBsAg, HBcAg, or HBeAg be demonstrated. CMV-EA was found in liver cell nuclei from three consecutive biopsies in OLT 2, preceding and coinciding with a rise in the titers of the complement-binding reaction, respectively (Figure 2d). FRPs were present inconsistently in the sinusoidal walls (Figure 5a) from the third

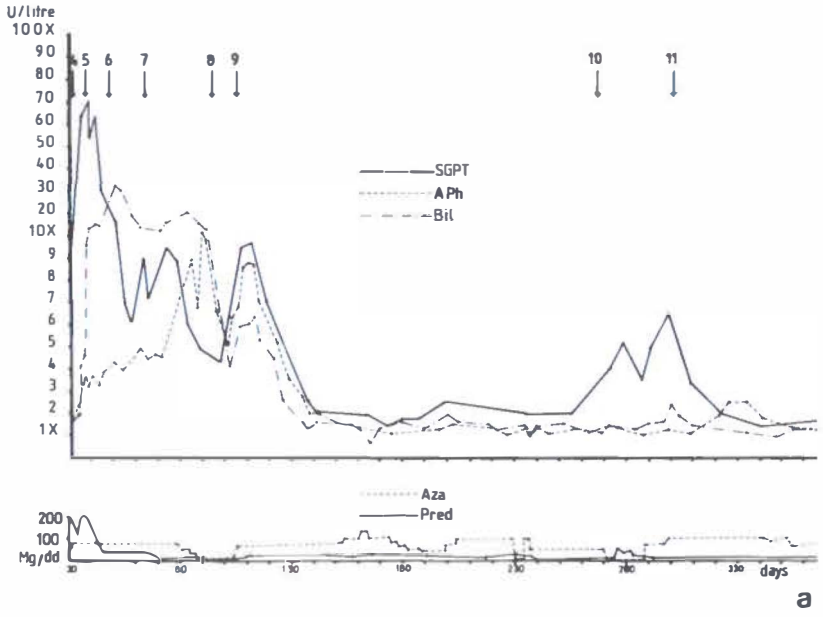


Figure 2. Serial biopsies with viral infection and subsequently acute graft rejection following forced withdrawal of immunosuppressive therapy (OLT 2). (a) Graph of some liver function tests *(upper part)* and the immunosuppressive regimen *(lower part)* from 30 days until 1 year after OLT. The numbered *arrows (upperpart)* indicate the taking of a nonprotocol graft biopsy. The histologic characteristics of Biopsies 4-7 were similar and have been interpreted as viral infection (see b, c and d); in Biopsies 10 and 11 a different histopathologic entity was present (see e). From about 90 to 105 days and from day 240 to day 260 no prednisone was given. *SGPT*, serum glutamic pyruvic transaminse; *APh*, alkaline phosphatase; *bil.*, total bilirubin; *Aza*, azathioprine, *Pred.*, prednisone. (b) Liver Biopsy 4 (see above). Acidophilic degenerated hepatocytes *(arrows)* are present in the parenchyma without any appreciable inflammatory infiltrate. (H&E, x 350). (c) Liver Biopsy 7 (see above). Overview, showing normal liver parenchyma and portal tracts *(arrows)* with minimal inflammatory reaction. (H&E, x 140). (d) Liver Biopsy 7 (see above). CMV-EA/LA antibodies directly conjugated to HRP. There is staining of scattered hepatocytic nuclei. The nuclei are of normal appearance or slightly enlagerd; no inclusion bodies are found. This pattern is an indication of CMV infection with expression of CMV-EA. (DAB reaction, x 560). (e) Liver Biopsy 10 (see bove). Enlarged portal tract *(asterisk)* with a mononuclear infiltrate and spillover on the adjacent parenchyma with piecemeal necrosis *(arrows)* The other parts of the liver parenchyma are normal. (H&E, x 140).

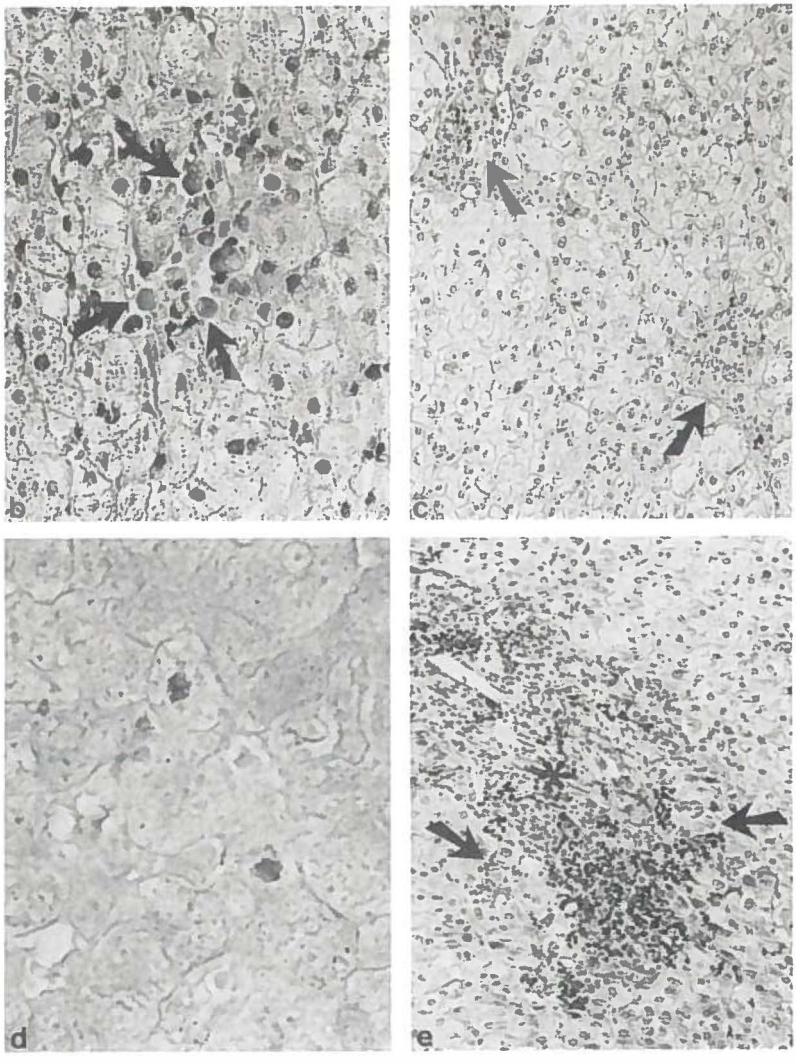

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Figure 3. Acute graft rejection (see also Figure 1c), nonprotocol biopsies. (a) Enlarged portal tract *(asterisk)* with infiltrate of mononuclear cells and spillover on adjacent parenchyma *(arrows)*. (H&E, x 140). (b) Detail of portal tract and periportal parenchyma. A denze inflammatory infiltrate, consisting of lymphocytes and plasma cells, is present in the portal tracts and invades the adjacent parenchyma with piecemeal necrosis *(asterisk)*. (H&E, x 350). (c) Overview, showing portal tract with inflammatory infiltrate *(asterisk)* and area with confluent necrosis in the parenchyma *(arrows)*. (H&E, x 140). (d) Detail of c, showing confluent necrosis of parenchyma *(asterisk)* and portal tract with lymphocytic infiltration of bile duct epithelium *(arrowheads)* and plasma cells in the infiltrate *(arrows)*. (H&E, x 350)

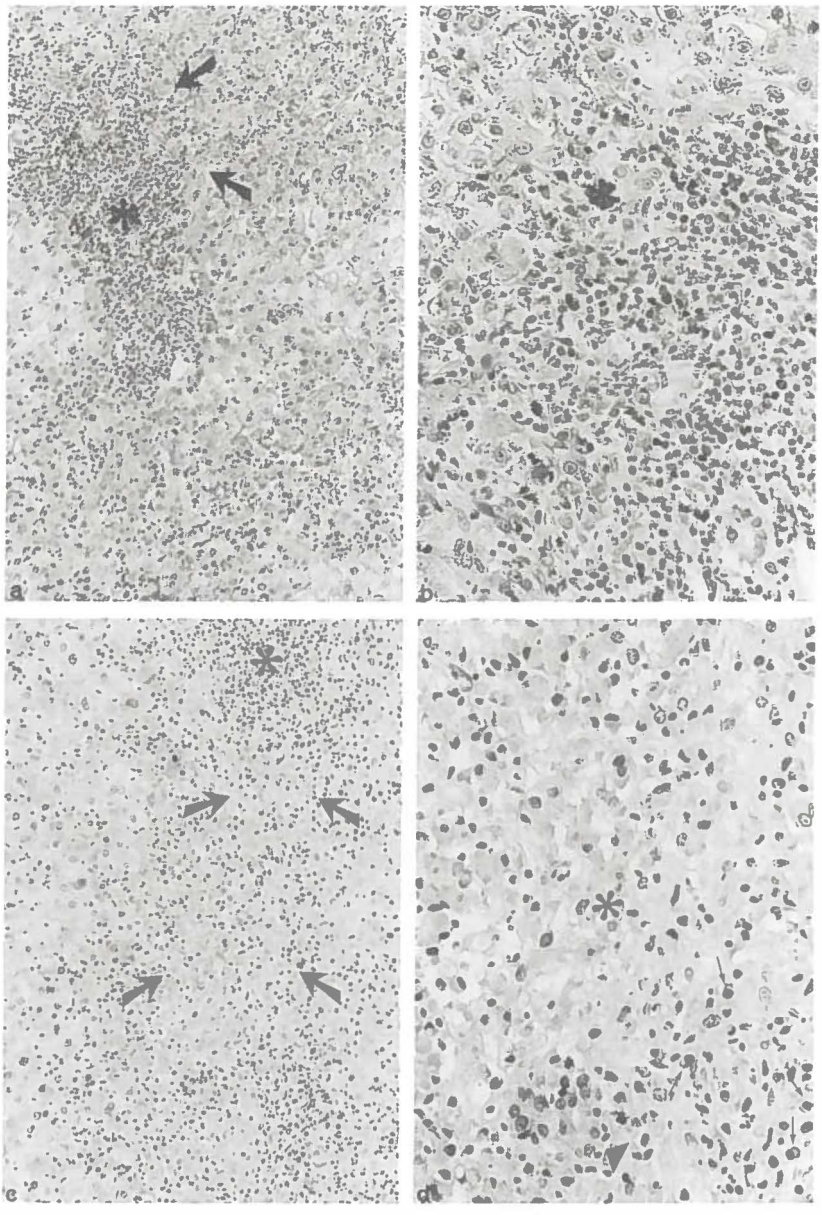
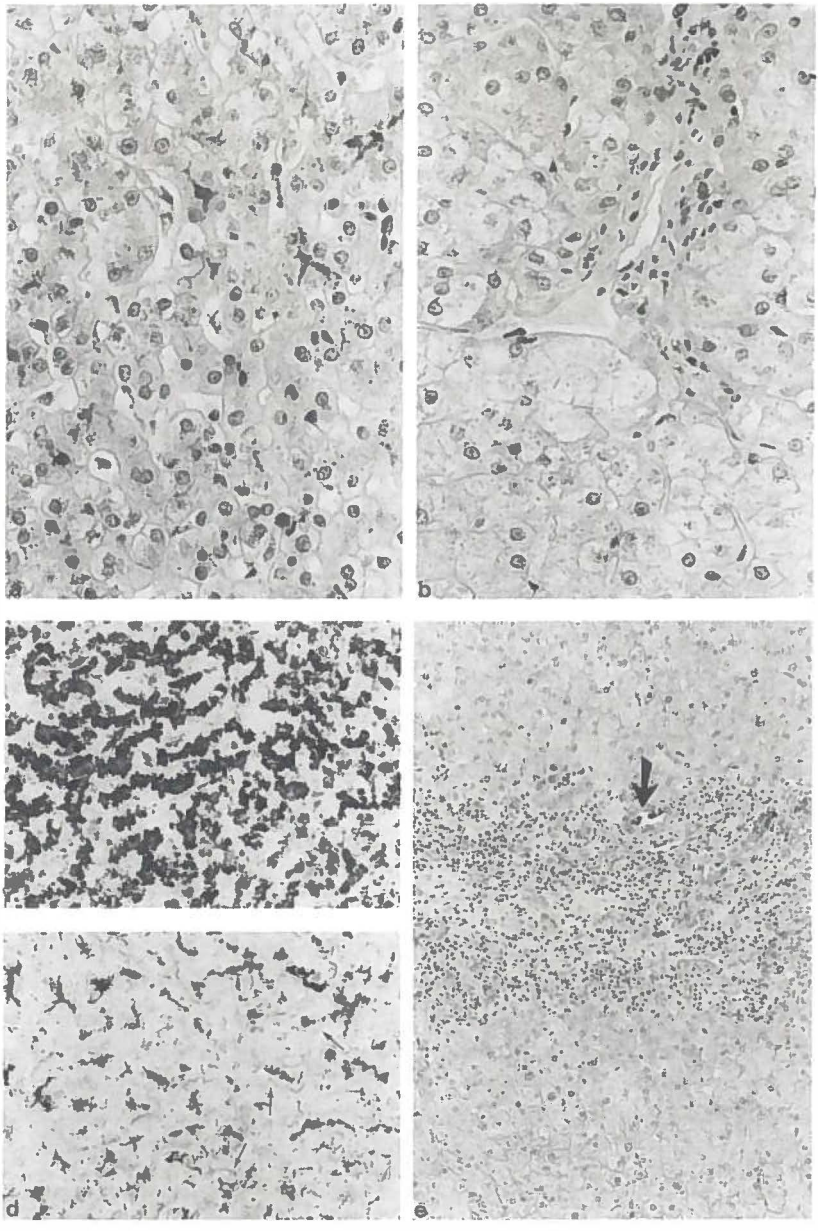


Figure 4. Cholestatic syndromes without morphologic evidence of rejection or viral infection: pure parenchymal cholestasis (a-d, same case) and bacterial cholangitis (e). (a) Liver parenchyma with accumulation of bile pigment in liver cells and canaliculi; there is no inflammation (H&E, x 350). (b) Same case, portal tract without inflammatory infiltrate. (H&E, x 350). (c) Same case. An increased acid phosphatase activity is demonstrable in the pericanalucular area of the hepatocytes. (Ac. Ph-ase, x 140). (d) Same case. The activity of leucine aminopeptidase in the pericanalicular area of liver cells is increased. Also, activity of LAP is present at the sinusoidal liver cell membranes *(arrows)*. (LAP, x 140). (e) Greatly enlarged portal tract with ductular proliferation and predominantly granulocytic infiltration. Bile pigment is present in intraductal concrement *(arrow)* and in the third acinar zone of the parenchyma (not shown). (H&E, x 140).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protocol biopsy onward, especially in the biopsy specimens with features of CALD. No layered fibrin deposits or intrasinusoidal microthrombi were found.

Enzyme Histochemistry

Liver cells lacking demonstrable NADH tetrazolium reductase activity were only found in areas with liver cell necrosis. Especially in the second protocol biopsy (1 hour after OLT), no loss of activity as an indication of irreversible degeneration could be demonstrated (Figure 1a and b). Acid phosphatase activity was increased in all graft biopsies from the third protocol biopsy onward; a particularly high activity was present in the biopsy specimens with pure parenchymal cholestasis and in the parenchyma adjacent to areas with liver cell necrosis (Figure 4c). Alkaline phosphatase activity, normally present in the portal tracts and the sinusoidal lining, was increased in activity and distribution during cholestasis. Leucine aminopeptidase activity (Figure 4d), normally confined to bile canalicular regions, showed an increase along the liver cell membrane in pure parenchymal cholestasis and in areas with necrosis. Mg^{2+}-dependent ATPase activity, normally seen in the portal tracts and in the periportal parenchyma, was increased throughout the parenchyma in conditions with cholestasis and necrosis.

Electron Microscopy

In 1-μ Epon sections some fine fat droplets were frequently present in hepatocytes, especially in graft biopsies taken during the first postoperative months.

In none of the biopsies viral particles were found. In the liver sinusoids, threadlike fibrin material could not be demonstrated conclusively in any of the biopsies. The Kupffer cells, especially in biopsies with features of CALD or pure cholestasis, showed reactive changes with extensive cell processes and many vesicles (Figure 5b). In the portal tracts, the endothelial lining of the blood vessels was swollen, especially during the first postoperative month; degeneration or necrosis of endothelial cells was not found. In biopsies with features of CALD, lymphocytes and monocytes were seen during extravasation from portal blood vessels, sometimes invading bile duct epithelium, and more frequently in the liver parenchyma adjacent to hepatocytes (Figure 5c).

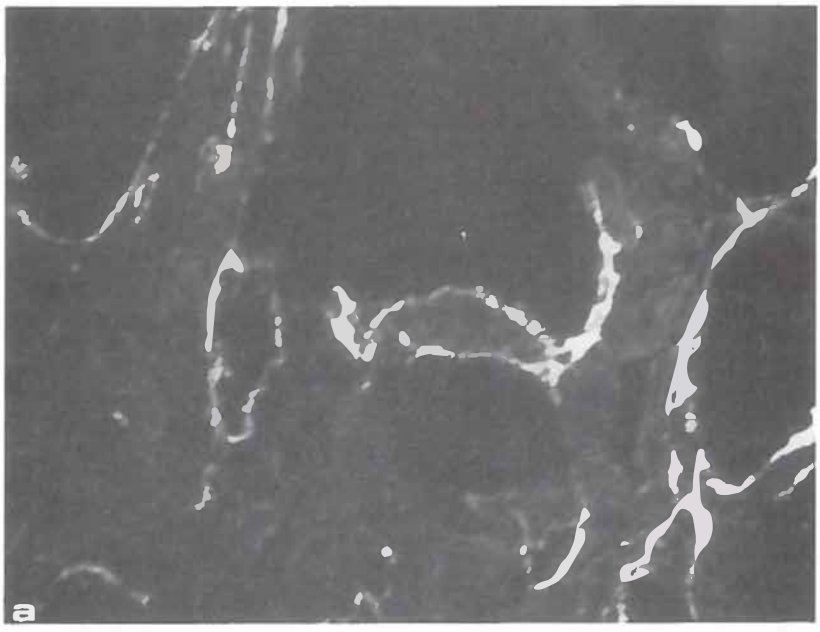

Figure 5a. Parenchymal reactive changes during phases of acute graft rejection. IF. Rabbit antibodies to FDP. Fibrin related products are present along the sinusoidal walls. Intrasinusoidal deposits are absent (x650).

DISCUSSION

The diagnostic relevance of liver biopsies during reversible functional disturbances of transplanted livers has not yet been fully documented. The early phases of the major graft syndromes and their mutually distinct histopathologic features are of diagnostic importance. Recently it was stated that histopathologic findings in graft biopsies of OLT may not provide an unequivocal answer to the question of whether or not graft rejection is involved, and that the diagnosis of rejection thus must be made clinically and by exclusion.[20] In contrast, the main results of this study are that in pathologic biopsy studies of liver grafts 1) a limited number of separate histopathologic entities do occur; 2) each of these entities closely resembles a well-known syndrome of liver disease in general (Table 2); and 3) most of these entities are closely related to clinical, functional, and serologic parameters. Each of these entities appears to be related to a circumscribed clinicopathologic syndrome and therefore is of diagnostic value.

The protocol biopsies served as a reference or 'preceding biopsy' for the evaluation of pathologic features in the unprotocol biopsies. Furthermore, the first protocol biopsies were used for the assessment of preexistant pathology in the donor liver (first biopsy) and changes due to graft preservation, pathology related to the surgery, and signs of hyperacute rejection (second biopsy). The other protocol biopsies were used for evaluation of signs of acute rejection, as reported to occur regularly[21] after the first week (third biopsy, see below), and signs of chronic rejection or the recipient's previous liver disease (biopsies at yearly intervals). In the only case with preexistent graft pathology, the enlargement of portal tracts remained evident in all subsequent biopsies, while the cholangitis and cholestasis disappeared during the first week after OLT; some reversible pathologic changes in donor livers thus seem not to interfere with the outcome of OLT. To evaluate the influence of graft preservation and surgery, the histochemical methods proved especially helpful; the viability of the graft, parameters of liver cell metabolism, the polarity of enzyme activities in hepatocytes (bile canalicular versus sinusoidal), and the characteristics of the acinar zones all appeared unchanged in every case. These fidings are in agreement with clinical investigations, demonstrating that the condition of the graft is not as critical in OLT as the preoperative condition of the recipient.[22]

Hyperacute rejection has never been observed, in accordance with the absence of second grafts in this series and with its reported absence in OLT.[4 23 24] It is of interest that hyperacute rejection was also absent in those patients with circulating liver membrane antibodies as a manifestation of their previous idiopathic autoimmune hepatitis[25]; this supports the evidence that circulating antibodies are not involved in liver rejection phenomena.[1 26]

The coagulative necrosis of liver parenchyma that has been observed in some cases during the early postoperative period seems to be related to preoperative and focal changes in circulation. These focal areas of necrosis were not related to definite changes in clinical condition or functional parameters. The absence of patholgy related to the recipient's *previous liver disease* is in accordance with the literature.[4] In the patient with primary hepatocelular carcinoma (OLT 3), the absence of recurrence can at least in part be attributed to the extensive preoperative screening for dissemination and the special precautions taken, such as the avoidance of a preoperative biopsy to exclude abdominal dissemination by needle-track contamination.[13 27]

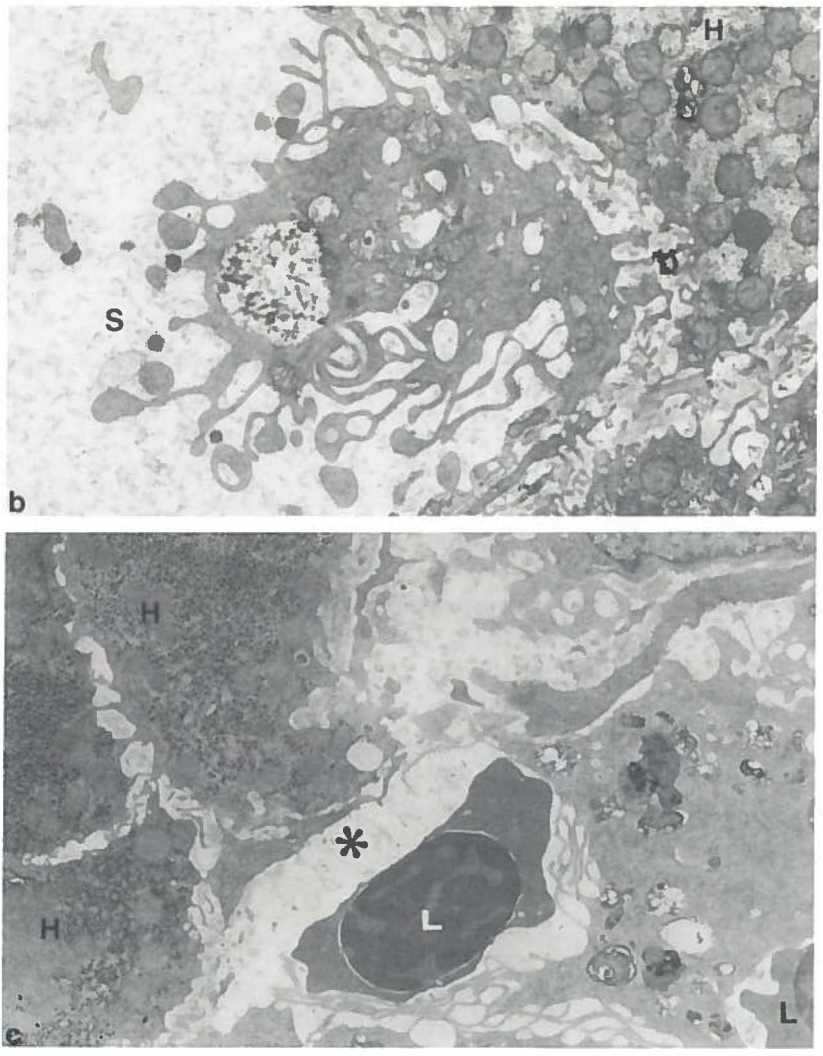

Figure 5 b-c. Parenchymal reactive changes during phases of acute graft rejection. (b) EM. Reactive Kupffer cell with many slender cell processes in the sinusoidal wall. S, sinusoidal lumen; D, Disse's space; H, Hepatocyte (x 7750). (c) EM. Lymphocytes (L) are present near the hepatocytes (H). Note the area of lysis *(asterisk)* adjacent to one of the lymphocytes (x 5420).

Because successive changes in the clinical status may occur quite regularly during the first months after OLT, the pathology in a biopsy taken because of such a change may reflect the end stage of preceding episodes, the onset of a

new one, or a combination of both. In this respect, a preceding graft biopsy proved most helpful to subtract changes already present for the evaluation of new pathologic features related to the new episode. For example, in OLT 2 and 3 during an episode of viral infection, the withdrawal of immunosuppressive therapy resulted initially in functional improvement, but subsequently a new episode of liver malfunction occurred. That this indeed was the onset of a new episode and not a sequel of the viral infection was proved by 1) the intervening symptom-free interval, 2) the subsequent improvement of clinical and biochemical parameters directly after raising the immunosuppressive therapy, and 3) different pathologic features in a new graft biopsy (see Figure 2). Thus, these episodes could be defined as acute rejection, induced by the forced withdrawal of immunosuppression.

Acute graft rejection was present in a number of biopsies; it proved always to be readily reversible and never contributed to ultimate graft failure. Morphologically three types of acute rejection could be distinguished. A first type consisted of a portal inflammatory infiltrate with lymphocytes, granulocytes, and plasma cells, without piecemeal necrosis or other involvement of the parenchyma apart from mild cholestasis in some cases. This type occurred exclusively in the third protocol biopsy and coincided with the second peak of liver function derangements, regularly present after one week without changes in the clinical regimen. All features of this mild type of acute rejection disappeared, as evidenced by normal portal tracts in the next biopsy. It has been tentatively interpreted as a delay of the effect of immunosuppression. A second type showed comparable features in combination with piecemeal necrosis; it thus showed all morphologic features of CALD. The concomitantly present fever rapidly disappeared following a moderate increase in the daily dose of prednisolone; the morphologic and functional changes proved reversible after a longer interval. A third type combined the features of CALD with confluent and/or bridging necrosis; and an inflammatory infiltrate in the parenchyma consisting of lymphocytes, plasma cells, and granulocytes. This type, comparable to severe CALD, was interpreted as uncontrolled acute rejection and occurred after forced complete withdrawal of immunosuppression; this type also proved to be readily reversible following an increase in the daily dose of immunosuppression as shown in the next biopsy after 1 or 2 weeks. Although some parenchymal cholestasis was occasionally present, a destruction of bile duct epithelium or other signs of cholangitis were inconspicuous, this in contrast to the findings of others.[21] The cellular

reaction and the striking similarities to idiopathic autoimmune CALD, together with the rapid therapeutic effect of prednisolone in both conditions, suggest that a similar cellular immune mechanism may be the main pathogenetic factor in acute rejection and autoimmune hepatitis. This hypothesis is further substantiated by an analysis of the lymphocyte subsets in liver biopsies in both conditions. [28] [29]

Chronic graft rejection, as the result of recurrent episodes of acute rejection or low-grade persistent rejection, could not be demonstrated in any of the biopsies. Endothelial proliferation, obliterative intimal changes of the portal blood vessels, and fibrous septa in the parenchyma were absent. Thus, it seems that the early recognition and rapid reversal of acute rejection phenomena has contributed considerably to the graft condition of recipients with survival times of 1 year or longer.

The pathology of *viral infection* in liver grafts is similar to that of viral hepatitis during immunosuppression in general: spotty liver cell necrosis without appreciable inflammatory changes in the parenchyma or portal tracts.[30] [31] Only CMV and HSV infections did occur, for all other potential viral infections none of the parameters tested ever became positive. HBV parameters, both serologically and in the biopsies, were negative in this series of OLT recipients. Although a positive pathologic diagnosis was supported by viral isolation or raised serum antibodies, conclusive evidence of viral particles in EM was never found, and in only one case could viral antigens (CMV-EA) be demonstrated in hepatocyte nuclei. One possibility for this discrepancy is a widespread viral infection with only minimal involvement of the graft; a more likely explanation might be that during these early phases of viral infection the expression of viral antigens was minimal. Both the pathologic changes and the clinical symptoms disappeared following a decrease or withdrawal of the daily immunosuppression dose. Chronic viral infections with prominent viral particles, as reported to occur during longstanding immunosuppression,[16] were not found.

Acute cholangitis occurred only in the course of bacterial infection. The infrequent occurence of cholangitis, both as a separate entity or in the course of acute graft rejection, is in contrast to other series.[4] [21] From the various factors that may have contributed to this difference, the surgical procedure with end-to-end choledochus anastomosis and special emphasis on optimal arterial blood supply seems the most important,[11] and the selective enteric decontamination[12] might be another factor during the first postoperative

months. The interpretation of the *'pure' parenchymal cholestasis* with no or minimal bile duct inflammation offers great problems. It was mostly associated with the recipient's death within 2 months, a poor preoperative condition of the recipient, pre- en postoperative coagulation disorders, abdominal hematomas not related to any of the surgical anastomoses and functional disturbances that were therapy-resistant. Because inflammatory cells or detectable immunoglobulins were absent, rejection seems improbable. Furthermore, bile flow was normal, and cholangiograms showed no disease. The enzyme-histochemical and ultrastructural changes in these cases were appreciable but can be found in any type of parenchymal cholestasis and thus seem to be consequences rather than causal factors. Because no ready explanation can be given, this syndrome in an otherwise still vital graft has been tentatively designated as functional failure. Comparable syndromes have been described in OLT.[4] The involvement of unrelated abdominal pathology such as the hematomas or disturbances in the enterohepatic cycle are factors that will be studied in comparable future cases.

The absence of hyperacute rejection and the relatively infrequent occurrence of acute rejection, both in morbidity and as a cause of death, emphasize the special role of the liver in immunology in general and in transplantation in particular. This is further evidenced by the protective effect of hepatic grafts on rejection of renal grafts[23] and by the lack of immune responsiveness following antigen administration in the portal circulation.[32] The impressive Kupffer cell activation present in the grafts during the first postoperative months, together with their special role in the clearance of particles and antigens from the portal blood,[33] suggest their participation in this protetive effect. The presence of FRPs in parts of the sinusoidal walls in immunofluorenscence without any ultrastructural evidence of fibrinlike fibrils may also be related to the retention of serum substances between the multiple slender processes of the Kupffer cells.

In conclusion, the main results of this study are as follows: 1) Acute rejection of liver grafts in OLT is a cellular immune reaction, morphologically similar to the autoimmune type of CALD; it occurs mainly as a result of low prednisolone dosage, is readily reversible, and is not frequently a cause of the patient's death. 2) Bile duct pathology seemed not to be involved in acute rejection and was never a serious complication; this difference, in comparison with other series, may be explained by the different surgical procedure with special care given the blood supply of the

bile ducts. 3) Based on the histopathology, a reliable diagnosis of acute graft rejection can be made. 4) Only a limited number of separate histopathologic entities occurs in liver homografts, each being of diagnostic value for a graft syndrome. 5) Protocol graft biopsies for the evaluation of graft condition at given times are helpful both for the assessment of clinically unsuspected pathology and as a reference for the interpretation of nonprotocol biopsies. 6) A graft biopsy may substantially add to the pathogenetic interpretation, differential diagnosis, and management of major graft syndromes in OLT recipients.

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CHAPTER 5

In situ analysis of mononuclear cell infiltrate in liver biopsies of patients with orthotopic liver transplantation

H.F. Eggink, H.J. Houthoff, S. Huitema, C.H. Gips* & S. Poppema.

Departments of Pathology and Internal Medicine*, University Hospital, Groningen, The Netherlands.

abstract

In this study the mononuclear cell infiltrate in liver biopsies of patients with orthotopic liver transplantation has been studied. It has been found, that in rejection as well OKT8+ as Leu3+ T lymphocytes are present.

keywords

Liver transplantation rejection lymphocyte-subsets

INTRODUCTION

Survival rate in patients with orthotopic liver transplantation (OLT) has been improved considerably in recent years. After OLT the patients are followed by biochemical, serological and histological methods. In a patient developing muscular pains, fever and deterioration of liver function the main possibilities are viral infection and rejection of the graft. Liver biopsies have proven to be of value in the differential diagnosis between these two possibilities (Eggink *et al.*, 1982).

In rejection the morphological picure of the liver resembles that of chronic active liver disease (CALD), i.e. piece-meal necrosis (PMN) with lymphocytes and plasma cells. In patients with renal allografts both humoral and cellular immune mechanisms have been implicated in the pathogenesis of graft rejection (Mc Phaul *et al.*, 1981; Strom *et al.*, 1975). The development of monoclonal antibodies directed against antigens expressed on subsets of mononuclear cells (Reinherz and Schlossman, 1981; Breard *et al.*, 1980) has facilitated the study of lymphocytes in peripheral blood and in the tissue. In patients with renal graft rejection an increased ratio of OKT4+ (helper/inducer) to OKT8+ (suppressor/cytotoxic) T cells has been found

in the peripheral blood, whereas in the kidney OKT8+ lymphocytes predominated (Cosimi *et al.*, 1981; Platt *et al.*, 1981). In this study the inflammatory infiltrate in liver biopsies has been analysed with the help of an immunoperoxidase technique employing monoclonal antibodies. The results will be compared with those in a previous study on the inflammatory infiltrate in liver biopsies of patients with CALD due to hepatitis B infection or idiopathic autoimmune hepatitis and of patients with primary biliary cirrhosis.

MATERIAL AND METHODS

OLT was performed in 12 patients. The previous liver disease was primary biliary cirrhosis (PBC, $n = 6$, all female, age 47-51 years), idiopathic autoimmune CALD (IA-CALD, $n = 2$, both female, age 54 years), cryptogenic cirrhosis ($n = 2$, 1 male, 1 female, 54 and 56 years) and primary hepatocellular carcinoma ($n = 2$, both female, 28 and 43 years). Liver biopsies ($n = 64$) were taken with a 1,6 mm Menghini needle and cut into 3 pieces. The largest part was fixed in 4% formaldehyde, embedded in paraplast and used for light microscopy and immunoperoxidase studies, the other part was immediately frozen in freon-22 and a small part was fixed in 2% glutaraldehyde for electron microscopy.

The lymphocyte subsets were characterized with hybridoma produced monoclonal antibodies from Ortho Pharmaceutical Corp. (Raritan, N.Y., U.S.A.) and the Leu 3 antibody from Beckton and Dickinson (Mountain View, Ca, USA) on frozen sections of liver biopsies. For characteristics of monoclonal antibodies see Table.

Table.

OKT1,3	mature, peripheral T lymphocytes	Reinherz and Schlossman, 1981.
OKT8	suppressor/cytotoxic T lymphocytes	Reinherz and Schlossman, 1981
OKM	monocytes, granulocytes, natural killer cells	Breard *et al.*, 1980; Kay & Horowitz, 1980
Leu 3	helper/inducer T lymphocytes	Ledbetter *et al.*, 1981

A two-step, indirect immunoperoxidase method was used. In short, sections were incubated with 25 µl of dilutions of the monoclonal reagents during 30 min. After repeated washing in PBS during 10 min., the sections were incubated with 25 µl horseradish peroxidase conjugated rabbit-anti-mouse immunoglobulin diluted 1:20 (Dakopatts, Copenhagen, Denmark),

supplemented with 1% human AB serum, during 15 min. The sections were washed in PBS during 10 min. and 3-amino-9-ethylcarbazole (Sigma, St. Louis, Mo, USA) was used as a substrate for the demonstration of peroxidase reactivity (Graham and Karnovsky, 1965). After counterstaining the sections were mounted with Gurr aquamount (Hopkin and Williams, Chadwell Heath, Essex, England). Plasma cells were demonstrated on 4 μ thick paraffin sections using peroxidase conjugated rabbit-anti-human IgG, IgM or IgA serum (Dakopatts, Copenhagen, Denmark). The sections were stained with diaminobenzidin (DAB) for 5 min.

RESULTS

In rejection ($n = 9$) a picture of CALD with PMN was seen. The majority of the lymphocytes were OKT1+, 3+ and thus of T cell origin. In the portal tracts, especially periportal and in areas with PMN, OKT8+ lymphocytes and in central parts of the portal tracts Leu3+ lymphocytes predominated. In the parenchyma both OKT8+ and Leu3+ lymphocytes were present.

OKM+ lymphocyte-like cells were inconspicuous in areas with PMN. Only few B lymphocytes were found. IgG and IgA positive plasmacells were present in most cases, often in areas with PMN, the IgG positive plasma cells predominated.

In patients with as previous disease PBC also some IgM positive plasma cells were present. Considerable numbers of polymorphonuclear leucocytes were present, especially in areas with PMN. Immunoglobulins at the surface of hepatocytes were not found. In viral infection ($n = 7$) the picture was variable. Most often there was spotty necrosis of hepatocytes with a moderate inflammation of the portal tract. There was no PMN. The majority of lymphocytes were OKT1+,3+,8+, only few Leu3+ lymphocytes were present. Some OKM+ lymphocyte-like cells were present in the parenchyma. B lymphocytes were absent, only few plasma cell were present, mainly in the portal tracts.

DISCUSSION

Rejection of the graft after OLT is not a common feature and develops mainly after forced withdrawal of immunosuppressive therapy because of viral infection.

The morphological picture of rejection of the liver resembles that of other types of CALD with PMN and plasma cells (Fig. 1.).

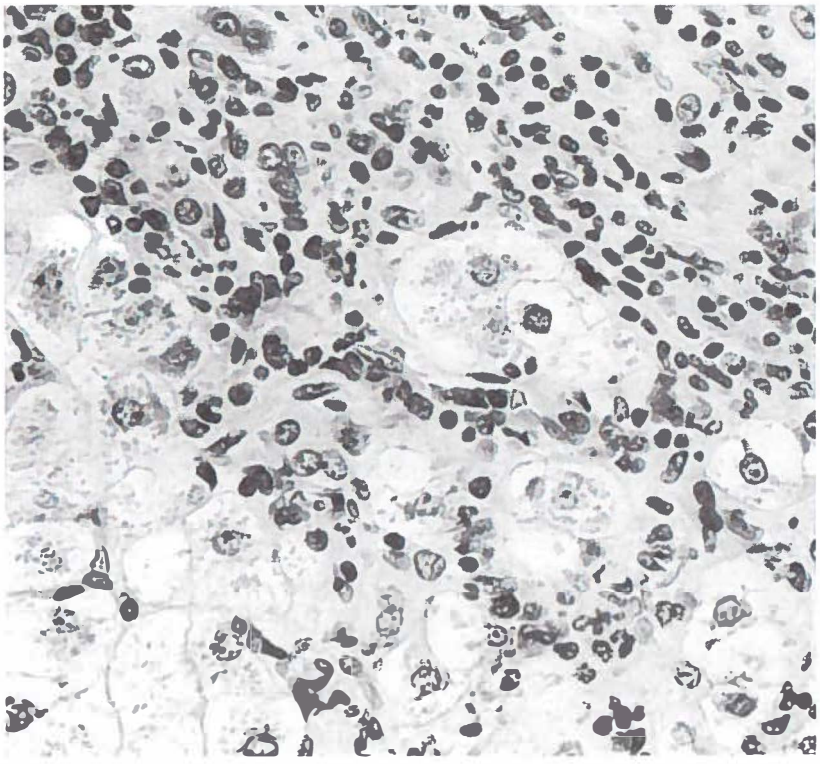

Fig. 1. Enlarged portal tract in rejection showing mononuclear cell infiltrate with piecemeal necrosis.

The purpose of this study was to establish whether humoral and/or cellular mechanisms are implicated in the pathogenesis of graft rejection and to compare the defined lymphocyte subsets in the liver in rejection with the lymphocyte subsets present in other types of CALD (Eggink *et al.*, 1982). The OKT8 antibody defines the cytotoxic/suppressor T cell population. In rejection OKT8+ lymphocytes predominated in areas with PMN, OKM+ lymphocyte-like cells and immunoglobulins at the surface of hepatocytes were absent. These findings suggest that liver cell damage in rejection is caused by T cell-mediated cytotoxicity. The absence of OKM+ lymphocyte-like cells and of immunoglobulins at the surface of hepatocyte argue against a role of killer and/or natural killer cells in rejection, such in contrast to IA-CALD where OKM+ lymphocyte-like cells are present in areas with PMN.

The Leu3 antibody defines the helper/inducer T cell population. In rejection Leu3+ lymphocytes predominated in the central part of portal tracts and in this respect resemble PBC, where also many helper cells in the portal tracts are present (Eggink *et al.,* 1982).

It is noteworthy that many helper cells were found during rejection not only in patients with PBC, but also in patients with other previous disease. Studies of peripheral blood of patients with rejection after OLT have not yet been published, but based on studies of peripheral blood of patients with PBC (Bhan *et al.,* 1982) and rejection of the kidney (Cosimi *et al.,* 1981) one may speculate that in rejection of the liver the ratio of OKT4+ to OKT8+ lymphocytes will increase, as a result of migration of OKT8+ lymphocytes from the peripheral blood into the liver.

In conclusion, it can be said that the inflammatory infiltrate in the liver during rejection resembles both IA-CALD and PBC. It can be speculated that the OKT8+ lymphocytes and the plasma cells are the results of a specific immune reaction against the hepatocytes, whereas the presence of Leu3+ lymphocytes in the portal tracts may be related to an immune reaction against bile-duct related antigens.

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CHAPTER 6

Liver membrane autoantibodies and the pathogenesis of liver diseases*

A serologic and immunohistologic study in patients with acute and chronic hepatitis, including cases before and after orthotopic liver transplantation.

Henk F. Eggink MD, Hendrik J. Houthoff MD PhD, Sippie Huitema BS, Chris H. Gips MD PhD, Sibrand Poppema MD

Departments of Pathology and Medicine, University Hospital and University of Groningen, Groningen, The Netherlands

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abstract

Liver membrane autoantibodies (LMA) have been thought to represent a pathogenetic factor for liver cell necrosis by antibody dependent cell mediated cytotoxicity in immune mediated liver diseases. This presumed role of LMA has been studied by comparison of its presence in serum samples ($n = 116$) with the phenotype of potential effector cell subsets in liver biopsies taken at the same time in eight patient groups, including liver homograft recipients.

Within the groups no difference in effector cell subsets was found between LMA+ and LMA− patients. Especially, subsets with a presumed non-T killer cell potential were present or absent according to the aetiology of the disease and irrespective of the presence or absence of LMA. In the follow-up of individual patients before and during therapy, the presence of LMA and the phenotype and activity of the inflammatory infiltrate varied without any interrelation. It is concluded, that LMA and effector cell subsets in the liver are two independent variables, suggesting that LMA is presumably not a pathogenetic factor in the immune attack of the liver, but an epiphenomenon of the activated immune system.

key words

autoantibodies lymphocyte subsets cytotoxicity hepatocytes hepatitis liver homograft

INTRODUCTION

The presence of serum autoantibodies (autoAb) to hepatocytic antigens in various acute and chronic liver diseases, including autoimmune diseases, is a

well-known phenomenon and a regularly used diagnostic tool (1-5). In addition to their diagnostic value, the autoAb to cell membrane antigens (mAg) of hepatocytes have been implicated in the pathogenesis of autoimmune liver diseases, based on the assumption that their binding to the mAg of an intact liver cell might constitute the first step in an antibody dependent cell mediated cytotxic (ADCC) reaction (6). The autoAb to an epitope of two membrane-related protein complexes, i.e. liver specific protein (LSP) and liver membrane antigen (LMAg), are regularly present in autoimmune liver diseases and have been studied both in vivo and in vitro for their possible pathogenetic implications (1, 6, 7). Of these, serum autoAb to LSP (anti-LSP) have been found in various liver diseases of unrelated pathogenesis (1-3) in serum titers related to the amount of liver cell damage (1, 3), resulting in the view that anti-LSP may represent a consequence rather than a causal factor of liver cell damage. On the other hand, the autoAb to LMAg (liver membrane autoantibodies, LMA) might represent such a causal factor in the piecemeal necrosis of periportal hepatocytes as part of an ADCC, as it is consistently associated with an autoimmune pathogenesis of liver disease, especially with idiopathic autoimune chronic active liver disease (IA-CALD) and primary biliary cirrhosis (PBC) (4, 5, 8).

For liver cell necrosis by ADCC to occur, the presence of serum autoAb as LMA should be related to an effector cell population with killer/natural killer (K/NK) potential in the areas with liver cell necrosis. Therefore, the aim of this study has been to test the presumed pathogenetic role of LMA by comparing, in various groups of liver diseases, the presence or absence of LMA in the serum with the phenotype of the potential cytotoxic effector cells in a liver biopsy, with serum samples and biopsies taken simultaneously from each patient. The presence and follow-up of both these parameters have been studied in patient groups with acute hepatitis (AH), chronic active hepatitis B (HB-CALD), IA-CALD before/during/after immunosuppressive therapy, PBC, orthotopic liver transplantation (OLT) following endstage IA-CALD or PBC, alcoholic liver disease (ALD) and drug-induced CALD. The main outcome of this study is the lack of any consistent relation between the presence or absence of detectable serum LMA on the one hand and the phenotype of the potential effector cell subsets in the areas with liver cell damage on the other, leading to the conclusion that a demonstrable pathogenetic mechanism of LMA in hepatocytic damage is lacking, LMA thus appearing to be an epiphenomenon of the immune reaction in patients with immune mediated liver diseases.

METHODS

Materials. Serum samples and liver biopsies (n = 116 each) from 73 patients with various types of liver diseases have been included in this study, as listed in table I. From each patient the liver biopsy and the serum sample were taken on the same day. In addition, 10 normal control sera from healthy blood donors, 10 normal control liver biopsies failing signs of the tentative clinical diagnosis sarcoidosis, and two LMA positive test sera (a gift from Dr. H.J. Schuurman, University of Utrecht, The Netherlands) from patients with IA-CALD were used.

All sera and biopsies used in this study were taken for the clinical evaluation of diagnosis or therapy. The diagnosis in all patients was fully documented with clinical, serological and histopathologic parameters. Material of some of the biopsies has already been used in other studies (9-12) from our group. According to diagnostic criteria the patients were listed in one of the eight groups as shown in table I.

Table 1. Clinical data, prevalence and immunoglobulin class of LMA and ANA in 73 patients with various liver diseases.

Group	pat. n=	sera n=	m/f	age	IT	LMA[1] IgG	LMA[1] IgM	LMA[1] IgG+IgM	ANA[1] IgG	ANA[1] IgM	ANA[1] IgG+IgM
HB-CALD	6	6	6/-	28-50	-	-	-	-	-	-	-
acute hepatitis	3	3	1/2	55-70	-	-	1	-	-	-	-
IA-CALD	13	16	2/11	16-68	-	10	1	1	8	1	-
IA-CALD treated[3]	5	14	-/5	16-56	5	3	1	-	1	1	1
PBC	15	18	2/13	40-79	-	-	5	3	-	2	-
alcohol-induced LD	9	9	3/6	23-65	-	-	2	-	-	2	-
drug-induced LD[2]	11	14	4/7	44-84	-	3	1	-	3	1	-
transplantation[3]	11	36	2/9	20-84	11	2	3	2	1	-	-
negative controls	10	10	9/1	25-55	-	-	-	-	1	-	-
postitive controls	2	2	-/2	23-44	-	2	-	-	1	-	-

Abbreviations: ANA, antinuclear antibody; f, female; HB-CALD, Hepatitis B chronic active liver disease; IA-CALD, idiopathic autoimmune chronic active liver disease; IT, immunosuppressive treatment (prednisone and azathioprine); LD, liver disease; LMA, liver membrane antibodies; m, male; PBC, primary biliary cirrhosis

[1] LMA-IgA was not detected in any of the patients; ANA-IgA was found in 3 cases of IA-CALD who showed also ANA-IgG.

[2] Comprises different drugs: nitrofurantoin, glafenine, phenylbutazone, ibuprofen and oxyphenisation.

[3] Patients have been followed up to 4 years.

The serum samples were directly frozen and stored at –20°C in a serum file. Samples were rapidly thawed directly prior to use. Apart from LMA, the samples were used for the detection of autoantibodies to smooth muscle (SMA), mitochondria (AMA), nuclei (ANA) and double stranded DNA. The percutaneous liver biopsies were taken with a Menghini needle of 1.6 mm diameter. A biopsy specimen of at least 4 cm was routinely obtained, from which parts were used for routine histology and immunoperoxidase (IP) studies on formalin fixed and paraffin embedded tissue, IP and histochemical studies on cryostat sections of fresh frozen tissue, and transmission electron microscopical (TEM) studies on tissue fixed in 2% buffered glutaraldehyde, postfixed in 1% OsO_4 and embedded in Epon.

Liver membrane autoantibodies. Serum LMA was demonstrated using an indirect immunofluorescence (IF) test on isolated hepatocytes in suspension as described in detail elsewhere (4). The hepatocytes were mechanically isolated from rabbit livers by perfusion in vitro with salt solutions without the use of hydrolases. Following several washings the viability of the cells was tested by vital staining with 0.1% trypan blue and viable cell suspensions were freshly used. Samples of the liver cell suspension were routinely incubated with patient sera in dilutions 1:2 and 1:10. Before use the complement in the sera was inactivated by heating for 30 min at 56°C. The liver cell suspension was incubated with the sera for 30 min at 37°C under continuous shaking. Following several washings with phosphate-buffered saline (PBS) the hepatocytes were incubated with fluorescein-conjugated rabbit-anti-human IgG, IgM or IgA serum (Dakopatts, Copenhagen, Denmark), diluted 1:40, for 30 min at 37°C under continous shaking. After washing in PBS, 25 μl of the cell suspension was applied onto a slide and examined the same day with a Leitz fluorescence microscope with epiillumination. The screening was always performed independently by the same two observers, using the normal control sera and the positive test sera as a reference. Serum ANA could be determined in the same preparations. The patient sera in the same dilutions were also used for the incubation of 4 μm cryostat sections of the patient's own and a normal rabbit liver biopsy in a comparable indirect IF technique. In each of these procedures, negative controls were prepared by substituting normal human serum for the patient's serum in an otherwise similar procedure.

Mononuclear subsets. Phenotyping of the lymphocyte subsets was performed in serial 4 μm thick cryostat sections using monoclonal antibodies of the Leu (Beckton and Dickinson, Mountain View, California

USA) and OKT (Ortho Pharmaceutical Corp., Raritan NJ, USA) series and a monoclonal antibody to human IgM. The characteristics and specificities of these monoclonals have been the subject of many reports (13-19), details on the indirect immunoperoxidase technique used are given in previous papers (9, 10, 12). In this study we used monoclonal antibodies anti-OKT1 and OKT3 for phenotyping all peripheral T lymphocytes, Leu3a and anti-OKT4 for helper/inducer T cells, anti-OKT8 for cytotoxic/suppressor lymphocytes, anti-OKT11 for E-rosette receptor bearing cells, anti-OKM1, 2 for a population of non-B non-T lymphocyte-like cells with K/NK potential together with monocytes and a population of granulocytes, Leu7 for a population of NK cells and anti-IgM for B lymphocytes. Plasma cells and immunoglobulins (Ig) at the surface of hepatocytes were demonstrated in 4 μm thick paraffin sections in a direct IP method using peroxidase conjugated rabbit-anti-human IgG, IgM and IgA serum (Dakopatts, Copenhagen, Denmark).

Scanning electron microscopy (SEM). Rabbit hepatocytes in situ and within 10 min after mechanical isolation were studied with SEM, both as part of the viability control and to check surface structures and cell polarity of hepatocytes in suspension. For the hepatocytes in situ, following a short perfusion with saline to flush the blood out of the liver, fixation was similar to that mentioned for the human material. The mechanically isolated hepatocytes were rapidly washed in PBS and fixed with 1% buffered paraformaldehyde for 5 min at room temperature. Following postfixation in 1% aqueous OsO_4, critical point drying in a high vacuum apparatus for 48 hr and a gold sputtered coating of 100Å thickness, the cells were used for SEM in a Jeol JSM-35C.

RESULTS

The presence and Ig class of LMA and ANA in the serum samples of the patient groups are listed in table I. The prevalence and serum titer of LMA was highest in the IA-CALD group. The combined presence of LMA and ANA, both of IgG class, was highly diagnostic for IA-CALD but was also present in the sera from two patients with a drug-induced hepatitis due to the laxative oxyphenisation. In the latter two patients, the histopathology and the phenotype of the lymphocyte subsets was also similar to those in the IA-CALD group. Both LMA and ANA could be identified in the same preparations of isolated hepatocytes in suspension (fig. 1), the LMA giving

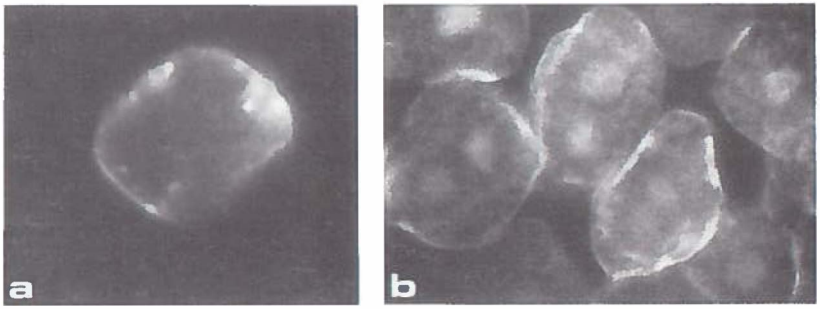

Fig. 1. Detection of liver membrane autoantibodies (LMA) by indirect IF on rabbit hepatocytes in suspension, x 40. (a) LMA of IgM class in serum of a patient with PBC (b) LMA of IgG class in patient with IA-CALD. Note the positive IF of nuclei, implicating the presence of ANA of IgG class as well.

a homogenous surface binding pattern with incidentally curvilinear areas of higher intensity resembling a bile canalicular pattern. On the contrary, on sections of fresh frozen liver, both of normal rabbit liver and of the patient's own biopsy, only the presence of ANA could be detected. No membrane-related or cytoplasmic localization of antibody binding could be identified in any of these preparations.

The differences between hepatocytes in situ and in suspension are shown in fig. 2. Even during the most rapid procedures, taking less than 5 min before fixation of the single cells in suspension, no surface structures resembling the original polarity of the hepatocytes, i.e. bile canalicular and sinusoidal microvilli, could be detected anymore in SEM. A rounded form with scattered microvilli all over the surface of the hepatocytes thus occurs rapidly during the process of preparing isolated liver cells.

In TEM, the ultrastructure of the potential effector cells in relation to hepatocytes in areas with piecemeal necrosis was studied. In all groups, including the liver grafts (11), the effector cells had the characteristics of lymphocytes (fig. 3).

The results of the phenotyping of the inflammatory infiltrate in cryostat sections (fig. 4) are summarized in table II. Serial sections were used for the demonstration of the lymphocyte differentiation antigens by a panel of monoclonal Ab, thus enabling the recognition of the phenotypes of the subsets by comparing for each of the monoclonals the regional differences in density of positively staining lymphocyte-like cells. For example the lymphocytes invading a given area of piecemeal necrosis in the liver

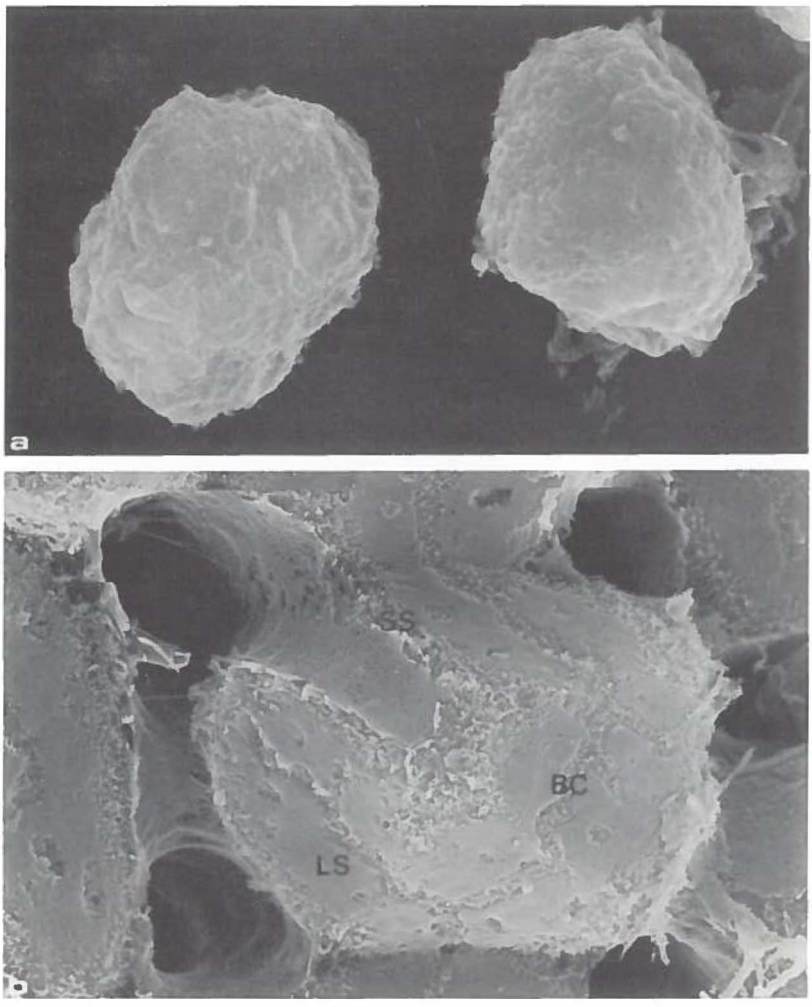

Fig. 2. Scanning EM of rabbit hepatocytes. (a) isolated hepatocytes in suspension, obtained within 5 min after isolation. Microvilli cover the cell surface, no evidence of the original membrane specializations *in situ* are retained. x 4,300. (b) fractured liver surface showing bile canaliculi (BC) with microvilli, smooth lateral surfaces (LS) and sinusoidal surfaces (SS) covered with numerous microvilli. x 3,400.

parenchyma of a biopsy could, by comparison of the serial sections, be phenotyped as belonging to a subset of OKT1 –, OKT3 –, Leu7 –, Leu3a –, OKT8 +, OKT11 +, OKM1 – cells (20). In the table this subset is represented as

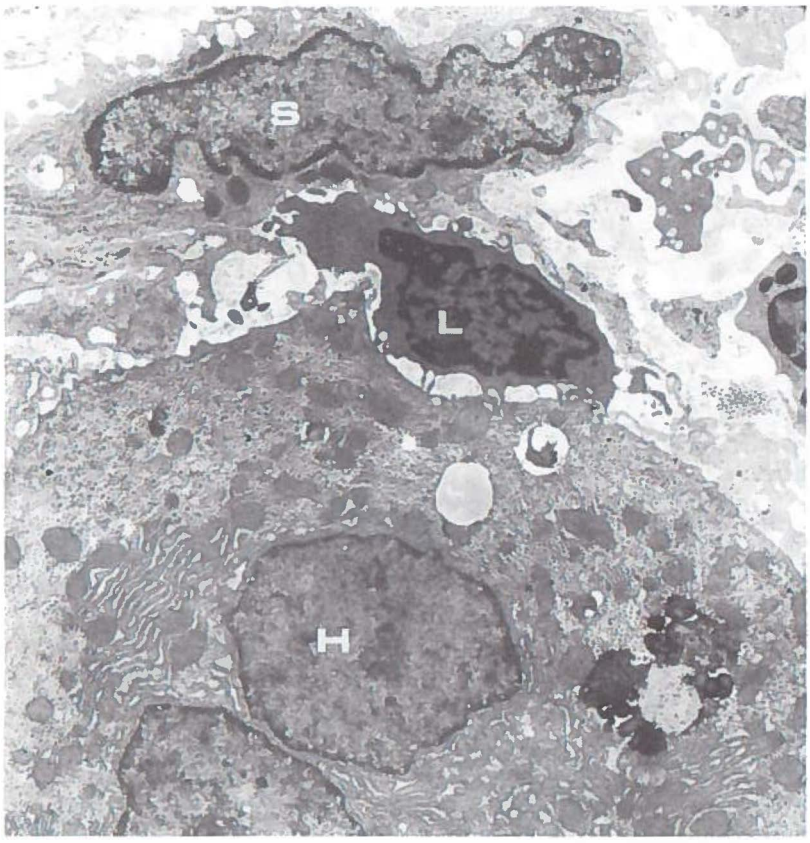

Fig. 3. Transmission EM of area with piecemeal necrosis, showing sinusoidal cell (S), hepatocyte (H) and lymphocyte (L) in the space of Disse. Note the slender processes of the lymphocyte in contact with the cell membrane of the hepatocyte. x 5,000.

Fig. 4. Immunohistological staining of the phenotype of lymphocyte subsets on serial cryostat sections of liver. OKT 3(a, d), OKT11 (b, e) OKT8 (c, f), x 350.
a-c. same area with piecemeal necrosis in LMA postitive patient with IA-CALD, showing the prevalence of a lymphocyte subset characterized by an (OKT3-8+11+) phenotype.
d-f. same patient, now LMA negative during immunosuppressive therapy. In serial sections of the same area with piecemeal necrosis, the prevalence of a lymphocyte subset with a similar phenotype is showed

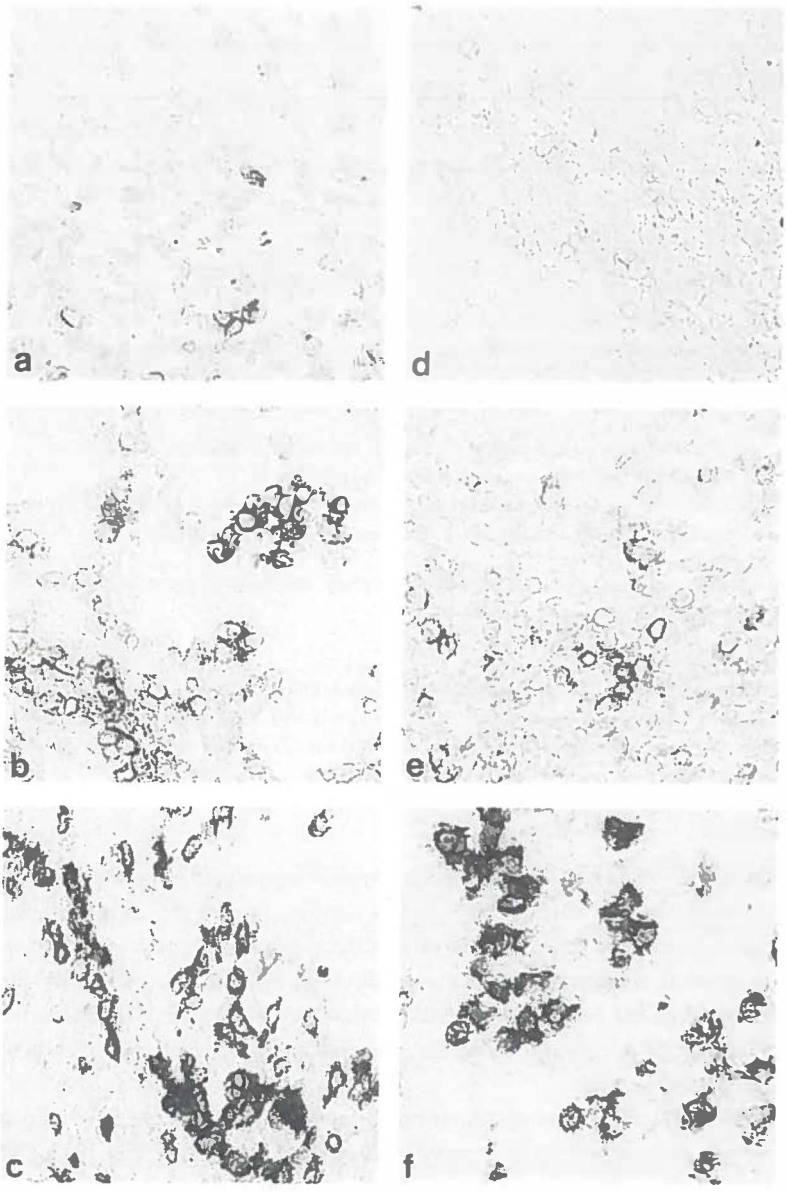
a
d
b
e
c
f

TABLE II

Distribution of the mononuclear cell subsets in liver biopsies of patients with various liver diseases.

Subsets	Functional interpretation	HB-CALD	AH	IA-CALD	IA-CALD-treated*	PBC	ALD	DLD	OLT***
OKT8+, 11+, 1+, 3+	cyt./supp. T cell	++	+	+	+	++	+	v	±
OKT8+, 11+, 1–, 3–	(?) cyt. non-T cell	–	++	++	++	+	±	v	++
OKM 1,2+	K/NK cell	±	–	+	+	±	–**	v	±
Leu 7+	NK-cell	–	+	–	–	±	n.d.	v	±
IgG, M, A	plasma cell	+	–	++	+	+	–	v	++

– = absent; ± = some; + = appreciable number; ++ = many

*) = immunosuppresive therapy with prednisone and azathioprine

**) = only many polymorphonuclear leucocytes were found

***) = acute rejection as the condition most resembling the morphology of IA-CALD or PBC has been included; in other OLT syndromes other subsets occur.

No differences in distribution of the lymphocyte subsets were found in any of the groups between LMA+ and LMA– patients.

Abbreviations used:
AH = acute hepatitis; ALD = alcoholic liver disease; cyt. = cytotoxic; DLD = drug induced LD; HB-CALD = hepatitis B chronic active liver disease; IA-CALD = idiopathic autoimmune chronic active liver disease; n.d. = not done; PBC = primary biliary cirrhosis; OLT = orthotopic liver transplantation; supp. = suppressor; v = variable.

(OKT1 – 3 – 8 + 11 +). For the patients within a group, the phenotypes of the lymphoid subsets were nearly always similar, but in the group of drug-induced hepatitis a considerable variability of subset phenotypes was found, thus making a semiquantitative evaluation in table II impossible. By comparison of table I and table II, it follows that the phenotype of the inflammatory infiltrate was found to be independent of the presence or absence of LMA in the serum.

In table III the follow-up of patients in the group of IA-CALD during and after immunosuppression treatment is shown. It appears that both LMA and ANA may remain present in detectable serum titers in the absence of inflammatory infiltrate in follow-up biopsies during and after therapy. On the other hand, the inflammatory infiltrate may remain of similar activity and phenotype long after the disappearance of LMA in the serum.

In the 5 patients that underwent OLT in the course of a LMA-positive IA-CALD or PBC, LMA reappeared in the serum of 3 OLT recipients after a LMA-negative interval of 1-3 months. As is shown in table IV, the reappearance of LMA coincided with various forms of graft pathology not related to original liver disease. The phenotype of the inflammatory infiltrate in the liver varied according to the form of graft pathology, it was fully comparable to the inflammatory infiltrate in similar forms of graft pathology in LMA-

TABLE III

The prevalence of LMA in patients with IA-CALD before and during immunosuppressive therapy, follow-up period of 4 years.

Patient	Sex	Age	Number of sera	before therapy ANA	before therapy LMA	during therapy ANA	during therapy LMA
1	♀	44	3	-	IgG	-	IgG
2	♀	48	4	IgG + IgM	IgG	-	-
3	♀	16	5	-	IgG	-	-
4	♀	56	4	-	-	-	-
5	♀	52	2	IgG	IgG	IgG	IgG

TABLE IV

Follow-up of LMA positive patients after orthotopic liver transplantation (OLT).

original disease	LMA (Ig class) before OLT	LMA (Ig class) after OLT	syndrome at seroconversion	follow-up
PBC	IgM	neg. until day 80, then IgG + IgM	acute rejection	alive after 3 yrs, no signs of PBC
PBC	IgM	neg. until day 40, then IgG + IgM	bacterial cholangitis	alive after 3 yrs, no signs of PBC
PBC	IgM	neg.	-	died after 10 days
IA-CALD	IgG	neg.	-	died after 20 days
IA-CALD	IgG + IgM	neg. until day 30, then IgM	acute viral infection	alive after 4 yrs, no signs of IA-CALD

negative transplant recipients. The phenotype of the inflammatory infiltrate in the graft during episodes of acute rejection, as the condition most closely resembling IA-CALD (11), has been included in table II. In these 3 LMA-positive OLT recipients, the seroconversion did not result in an unfavourable follow-up or recurrence of the original disease during a mean follow-up period of 3 years.

DISCUSSION

An antibody dependent cell-mediated cytotoxic reaction has been postulated as the main mechanism for hepatocytic lysis (piecemeal necrosis) in immune mediated liver diseases (6), based upon the characteristics of the inflammatory infiltrate in the liver and the presence of autoAb in the serum (1-5) supplemented with the data of in vitro studies (8, 21). For such a reaction to occur, both serum autoAb to hepatocytic mAg and lymphocytes or monocytes with K cell potential should be present at the site of liver damage. Two autoAb to hepatocytic mAg that have been implicated in the humoral part of an ADCC are anti-LSP and LMA. The supposed pathogenetic role of anti-LSP was virtually discarded following the finding that high anti-LSP serum titers were found in most liver diseases with ongoing liver cell necrosis irrespective of the aetiology, and furthermore by the reported relation between the amount of liver cell damage and the titer of anti-LSP (1, 3). The perspectives for a pathogenetic role of LMA were better, since the presence of LMA was found to be related to immune-mediated liver diseases as IA-CALD and PBC (4, 5, 8). The results with LMA in this study (table I) corroborate this relation, as LMA was perdominantly found in the serum of patients with IA-CALD and its follow-up (immunosuppressive treatment, OLT), PBC and allergic drug reactions mimicking IA-CALD. The determination of the titer and Ig class of LMA in serum is thus a useful contribution to the diagnostic tests for immune-mediated liver diseases. However, other autoAb as i.e. to smooth muscle, nuclei and mitochondria may also be present in these diseases, and although their precise function is incompletely understood, it is generally believed that their presence is not related to the pathogenesis of tissue damage but probably reflects the activity and dysregulation of the immune system in the patients. Thus LMA might merely reflect an activated immune system in immune mediated liver disease or alternatively might constitute a pathogenetic factor. To prove the latter, Ig of LMA type should be present at

liver cell membranes in areas with piecemeal necrosis together with K cells. The *in situ* localization of Ig at liver cell membranes could not be documented in our material. However, the methods used may have lacked sensitivity and thus do not rule out completely the *in situ* presence of Ig. Study of the presence of LMA in serum or of Ig *in situ* does not give conclusive evidence about its implications for the pathogenesis.

Lymphocytes with a phenotype compatible with K cell potential (OKM1+, Leu7+ or OKT8+ non-B non-T cells) were present in variable amounts in the liver tissue of the groups tested, but only in areas with piecemeal necrosis of IA-CALD they were prominently present. The lymphocyte subset characterized by the phenotype OKT1–3–8+11+ was most prominent in IA-CALD and although a non-B non-T cell subset by its lack of pan T cell markers, it is at present an unsolved problem whether this is a subset of activated T cells that have lost some of their pan-T cell markers (22, 23) or whether these cells constitue a non-B non-T cell subset representing for instance large granular lymphocytes (24, 25). As HLA-ABC backbone antigen is prominently present on hepatocytes in areas with piecemeal necrosis of IA-CALD (26, own unpublished observations), the concomitant presence of an activated T cell subset might indicate the presence of HLA-ABC restricted T-cell mediated cytotoxicity without the need of any humoral factor in the pathogenesis. On the other hand, the presence of an activated non-B non-T cell subset with K-cell potential, in conjunction with the presence of many plasma cells and B lymphocytes might indicate the presence of an ADCC without the need of any HLA expression on the hepatocytes. In much the same way, the activation of HLA-DR backbone antigen on hepatocytes and bile duct epithelium in PBC (onw unpublished observation) coincides with the presence of a subset with OKT1+3+4+8–11+ phenotype; this might indicate a HLA-DR restricted cytotoxic reaction of this subset as has been described (27), but an antibody dependent reaction is another possibility with B cell activation by a helper cell population of this phenotype. Study of the phenotype of potential effector cell populations does not give conclusive information about the pathogenetic mechanisms involved, as also concluded by others (28). This also implies that the less monoclonal antibodies are used, the less information about the phenotype of the subsets is obtained (20,29,30).

Although either the presence of LMA in the serum or the potential effector cell phenotype in the liver alone dit not yield conclusive evidence of the pathogenetic mechanisms, their interrelations might do so. The effector

cell populations in the liver parenchyma prove to be highly dependent upon the aetiology of the group as a whole and irrespective of the presence or absence of detectable serum LMA (compare tables I and II). This lack of consistency between the presence of LMA and the phenotype of the mononuclear subsets was further documented by (a) the absence of a detectable subset phenotype correlating with the presence of LMA, irrespective of the patient groups; (b) the follow-up of individual patients with IA-CALD during remissions and exacerbations, with a lack of correlation in appearance/disappearance between LMA in the serum and lymphoid subsets in the liver; and (c) the reappearance of serum LMA in patients after OLT without any consequence for the follow-up in general and for reoccurrence of IA-CALD in particular. To our opinion, the results with the OLT group in particular demonstrate quite clearly that the reappearance of LMA during a decrease in the immunosuppressive regiment (31) reflects the activity of the immune system as a whole and is not related to the pathogenesis of IA-CALD *per se*. In this respect, caution should be taken in the interpretatio of CALD-like lymphoplasmacellular infiltrates in a graft in combination with serum autoantibodies as an indication of recurrent IA-CALD or PBC in OLT recipients (32, 33); the autoantibodies reappear anyway in most cases and the lymphoplasmacellular infiltrate usually represent episodes of reversible acute rejection or sometimes also chronic rejection of the graft, as has also been pointed out elsewhere (11, 12). Thus, study of the interrelations between serum LMA and the phenotype of potential effector cell subsets result in the conclusion that LMA is presumably not a pathogenetic factor in immune mediated liver diseases but an epiphenomenon. However, the problem of the pathogenetic mechansims of immune attack in the liver, whether a T-cell mediated cytotoxicity or an ADCC, remains unsolved.

Although *in vitro* studies have demonstrated the possibility of an ADCC as the pathogenetic mechanisms in IA-CALD (4, 5, 7), caution should be taken in the translation of *in vitro* studies to the *in vivo* situation.

In the first place, single hepatocytes as target cells are fundamentally different from the hepatocytes *in situ;* the changes in the polarity of the cells as demonstrated in SEM and the changes in expression of mAg on the surface as demonstrated by the exclusive binding of LMA on single hepatocytes may modulate the target-effector cell interactions. In the second place, the HLA-restriction of T-cell mediated cytotoxicity interferes with a target-effector cell interaction if the target cells are not autologous, as i.e.

with rabbit hepatocytes or Chang cells. Thus, to settle the problem of the pathogenetic mechanisms involved, by *in vitro* methods, a short culture of the patient's own hepatocytes with restoration of the original polarity (34) may constitute an optimal target to evaluate the effector cell subsets and the immune mechanisms involved. This approach is currently studied by our group.

In conclusion, the results of this study confirm the value of LMA as a diagnostic test in immune mediated liver diseases, it is shown that LMA in serum and the mononuclear subsets in the liver are two independent variables in these diseases, it is deduced that LMA is presumably not involved in the immune attack of the liver, and it is stated that the problem whether a T-cell mediated or an antibody dependent K-cell mediated cytotoxicity is at the basis of the immune attack in these diseases remains still essentially unsolved.

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CHAPTER 7

General discussion

INTRODUCTION

CALD represents a disease state which may be caused by different aetiologic factors, both endogenous and exogenous, but with as a common morphological picture in the liver lymphocyte spill-over and piecemeal necrosis (Ch 1, 1, 2). The heterogeneity of the underlying causes of CALD is reflected by for instance the different course of the individual diseases and the different clinical response to therapeutical regimens (3, 4). Several facts suggested that the interactions of immunoregulation, whether as cause or as effect, may influence the prognosis in many liver diseases (5, 6). The early evidence favouring an important role for immune mechanisms in various liver diseases was based on serological or humoral abnormalities, in particular changes in serum levels of various immunoglobulins and the occurrence of serum autoantibodies. The importance of cell-mediated immunity is emphasized by the morphologic appearances in the liver, the changes in peripheral blood lymphocyte subsets and the demonstrable sensitization of lymphocytes to viral antigens and to hepatocytes or hepatocellular constituents (7-9). While the morphological presence of lymphocytes and plasma cells in many liver diseases suggested that an immune mediated reaction is occurring, the precise mechanisms of the liver cell injury remained uncertain.

Disturbances of immunity have in particular been sought to explain the chronic course which some of these diseases pursue in the susceptible host. The availability of measures to selectively influence either humoral or cell-mediated immunity would offer the opportunity to correct the abnormal response which leads to CALD. The success of such a venture requires a thorough understanding of the altered immunity that underlies a failure to recover from CALD. As long as this information is not available, attempts to use a specific immune modulating therapy are speculative. Without exact insight in the pathogenesis it remains difficult to make a choice between a common immunosuppressive treatment or rather a specific immunomodulating therapy or neither of both but, as in chronic viral infection, an antiviral therapy.

From *in vitro* studies with peripheral blood lymphocytes it has been made

apparent that cell-mediated cytotoxicity may be important for liver cell damage (10-12), however such assays may not be relevant to cytotoxic mechanisms *in vivo*. In addition, studies of peripheral blood lymphocytes may not reflect tissue findings as is reported in chronic liver disease (13, 14) and also in various autoimmune diseases in general. A diminished number of a given lymphocyte subset in the peripheral blood may reflect an absolute decrease of this subset, or of a relative decrease due to homing of these cells to the target organ. For example, in sarcoidosis the ratio of T-cell subsets was strikingly different in peripheral blood, pulmonary tissue, and sputum in the same patient (15).

It was natural, therefore, that we focused our attention on the analysis of the mononuclear cell infiltrate in the liver parenchyma as target organ, rather than in the peripheral blood. Another advantage of this approach was that not only an overall picture could be obtained of the lymphocyte subsets present in the liver, but it also allowed for the characterization of the lymphocytes actually attacking liver cells: the presumed effector cell subset.

LYMPHOCYTE SUBSETS

For the characterization of the lymphocytes monoclonal antibodies were used which react with differentiation antigens present on the surface of lymphocytes, characterizing distinct lymphocyte subsets with a functional correlate (16-19). At the start it was thought that lymphocytes within a subset were unique with respect to their functional repertoire and that the specific program of a certain subset of lymphocytes was linked to the expression of a particular cell surface antigen. However, it appeared that within a lymphocyte subset - characterized by only one monoclonal antibody for a specific surface antigen - lymphocytes were present with the coexpression of other and mutually different surface antigens and also with functional heterogeneity (17). Therefore the phenotype of a given lymphocyte subset may rather be judged by the application of a panel of monoclonal antibodies than by a limited selection (Ch. 3: addendum).

In this study the lymphocytes in liver biopsies were characterized using monoclonal antibodies from the OKT and Leu series. Literature data and specificities of these monoclonals are described in the preceedings chapters. By the application of a number of monoclonal antibodies on serial sections of liver biopsies the phenotype of the lymphocyte subsets in different groups of liver disease were described. The main findings are summarized in table I:

Table I

phenotype subset		subset in short		present in
OKT1 + 3 + 4 + 8 - 11 +	OKM - Leu7 -	OKT4+	Tcell	PBC, Rejection
OKT1 + 3 + 4 + 8 + 11 +	OKM - Leu7 -	OKT4+8+	Tcell	PBC
OKT1 + 3 + 4 - 8 + 11 +	OKM - Leu7 -	OKT8+	Tcell	HB-CALD, PBC
OKT1 - 3 - 4 - 8 + 11 +	OKM - Leu7 -	OKT8+	non-Tcell?	IA-CALD, AHB, Rejection
OKT1 - 3 - 4 - 8 - 11 +/-	OKM + Leu7 -	OKM+	non-Tcell	IA-CALD
OKT1 - 3 - 4 - 8 - 11 +/-	OKM - Leu7 +	Leu7+	non-Tcell	AHB

The OKT4 + 8 + T cell population found in PBC (Ch. 2) probably represents peripheral but rather immature T cells. Their significance is still not understood. The same subset is described in the mononuclear cell infiltrate in malignant melanoma (20) and myasthenia gravis, in the latter these cells are reported to disappear after thymectomy (21).

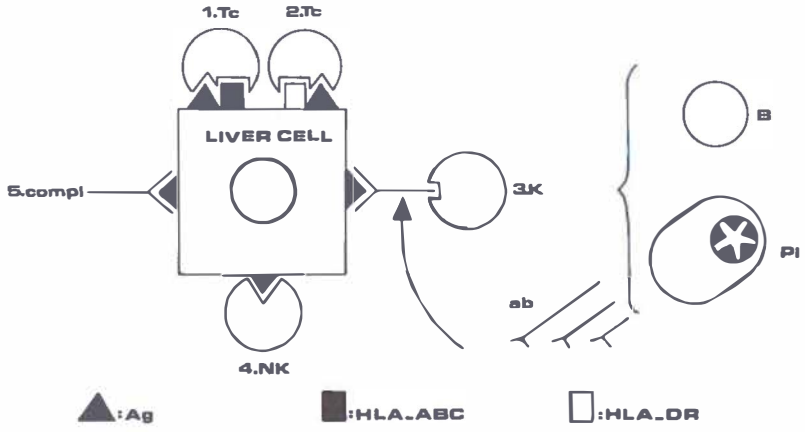
From table I it is obvious that different non-T cell subsets with potential K/NK activity exist with different phenotypes (Ch. 2, 6) and occur in different types of CALD, predominantly in areas with liver cell necrosis. The lymphocyte subset characterized by the phenotype OKT1 - 3 - 8 + 11 + was most prominent in IA-CALD (Ch. 2). Although a non-B non-T cell subset by its lack of pan-T cell markers (OKT1 + 3 +), it is at present an unsolved problem whether this is a subset of activated T cells that have lost some pan-T cell markers (22, 23), or whether these cells constitute a non-B non-T cell subset representing for instance large granular lymphocytes (24, 25).

IMMUNE MECHANISMS INVOLVED IN LIVER DISEASE

The possible immune mechanisms responsible for liver cell damage are represented in table II.

The study of the immune mechanisms involved in the cytotoxic reaction in the target organ implies the detection of the features of one of these mechanisms, as summarized in table II. MHC antigens (HLA determinants) are known to be enhanced in areas with piecemeal necrosis (26, 36), in most, if not all types of CALD. Mostly the presence of MHC *class I* antigens (HLA-ABC) on liver cells is enhanced, the corresponding MHC class I restricted T cells involved in a cytotoxic reaction are OKT8 + (27). MHC *class II* antigens (HLA-DR) are enhanced on liver cells and bile duct epithelium in some conditions including PBC (36), the corresponding MHC class II

Table II: Possible immune mechanisms responsible for liver cell damage.



1. specific T cell mediated cytotoxicity, MHC class I (HLA-ABC) restricted
2. specific T cell mediated cytotoxicity, MHC class II (HLA-DR) restricted
3. antibody dependent cellular cytotoxicity
4. natural killer cell cytotoxicity
5. antibody dependent complement activation

Abbreviations used: ab = antibodies; Ag = antigen; B = B lymphocyt; compl = complement; K = killer cell; NK = natural killer cell; Pl = plasma cell; Tc = cytotoxic T cell.

restricted cytotoxic T cells are known to be OKT4 + (27). In antibody dependent cellular cytotoxicity (ADCC) and natural killer cell (NK) cytotoxicity no HLA expression is required, but non-T cells in the presence or absence of a specific antibody are needed for a cytotoxic reaction. In complement mediated cytotoxicity, neither cytotoxic cells nor HLA expression are required.

The circumstantial evidence that a cytotoxic reaction is indeed of a given type depends on the presence in the target organ of the pertaining features (table II) and also on the absence of the features of other possible immune mechanisms.

In many publications on these topics parts of this statement have not been taken into consideration and only the *presence* of some features has been taken into account as evidence for a given type of cytotoxic reaction. Thus it is obvious that results with peripheral blood lymphocytes or serum, for

example in determining lymphocyte subsets (13, 14), sensitization in vitro of lymphocytes to hepatic antigens (10-12) or the presence of liver specific autoantibodies in serum (28,29), are not necessarily elucidating the type of cytotoxic reaction in the liver responsible for target cell damage. If the studies are extended to include also the liver as target organ, results have been obtained on the presence of immunoglobulins (30), on activation of MHC determinants (26), on only some parameters for phenotyping mononuclear cell populations and their subsets (31, 32, 33), or on viral antigenic markers. However each of the foregoing results *per se* is not allowing for definite statements on the pathogenetic mechanisms involved. Only studies including all of these parameters, where the presence of some of them is enhanced by the absence of others, are likely to elucidate the mechanisms involved.

PATHOGENETIC MECHANISMS ACCORDING TO AETIOLOGY

In the various forms of hepatitis B (Ch. 2, 3), the foregoing is best illustrated. In acute, self-limited hepatitis B phenotyping of the potential cytotoxic effector cells showed a preponderance of non-B non-T lymphocytes with an appreciable number of NK-like cells (Leu7+). Other cell types (B cells, plasma cells, T lymphocytes) were conspicuiously absent, as were immunoglobulins in situ. Presumably, this type of immune reaction (type 4 in table II) leads to an adequate clearance of virally infected hepatocytes, viral antigens are only sporadically detectable and cytopathogenic effects (ground-glass cells) are absent.

In chronic hepatitis B, the inflammatory infiltrate varies from virtual absence of lymphocytes (carrier state) to many lymphocytes with PMN in HB-CALD. Here, in the areas with PMN OKT8 + T cells are the main cell type in combination with an enhanced expression of HLA-ABC on hepatocytes. As B cells, plasma cells, non-T lymphocytes and immunoglobulins are conspicuiously absent, an MHC class I restricted cytotoxic T cell reaction (type 1 in table II) is the only possible immune mechanism, both on positive and negative arguments. As the virus usually is not cleared in HB-CALD, this is an obviously less adequate immune reaction. This results in large amounts of viral antigens with cytopathogenic effects (ground-glass cells) throughout the parenchyma. As HB-CALD may be interpreted as the persistence of an early stage of acute hepatitis B (34), a defective activation of a NK cell subset in early stages of hepatitis B may be a major factor in the persistence of hepatitis B viral infection.

In IA-CALD (Ch. 2, 6), many plasma cells and T cells are present in the portal tracts, in the areas with PMN the potential effector cell population consists of a minor population of T cells (OKT1 + 8 +) but mainly of non-T cell subsets (OKT1 - 8 +, OKM +). The presence of in situ immunoglobulins was an inconstant finding, but a lack of sensitivity of the in situ detection can not be excluded. Autoantibodies including LMA were frequently present in the serum; however, no correlation could be established between the presence or absence of LMA in the serum and the activity/phenotype of the potential effector cells in the liver. Enhanced expression of HLA-ABC is present on hepatocytes in the areas with PMN in IA-CALD, as in all other conditions with PMN (26, 35). No unequivocal immune mechanism can be deduced from these results, as the parameters allow for at least two of the possible mechanisms (table II): most probably an antibody dependent cell mediated cytotoxicity (type 3, but not involving LMA as autoantibody) but T cell mediated cytotoxicity (type 1) can not be excluded.

In PBC (Ch. 2, 6), both bile duct epithelium and hepatocytes are targets for the immune reaction. Many plasma cells, variable numbers of B cells and several T cell subsets (OKT1 + 4 +, OKT1 + 8 +, OKT1 + 4 + 8 +) are present in the portal tracts, the OKT8 + T cell subset is especially prominent in the periphery of the portal tracts and the adjacent areas with PMN. Non-B non-T cell subsets (OKT1 - 8 +, OKM +) were present in small numbers mainly in the parenchyma. For immunoglobulins and LMA, a similar statement as in IA-CALD can be made. Apart from some HLA-ABC expression, enhanced expression of HLA-DR is present on hepatocytes, bile duct epithelium and small proliferating bile ducts (36). Again, no unequivocal immune mechanisms can be deduced from these results. In the areas with PMN, a T cell mediated cytotoxicity, both MHC class II (type 2 in table II) and MHC class I (type 1 in table II) restricted, is possible, but an antibody dependent cell mediated cytotoxicity (type 3 in table II) cannot be excluded completely. For the bile ducts as target organ, a MHC class II restricted T cell cytotoxicity (type 2) is most probably the immune mechanism involved, but again a humoral component can not be ruled out completely.

In episodes of acute rejection of liver homografts (Ch. 4, 5, 6) the phenotyping of lymphocyte subsets was not particularly helpful in elucidating the immune mechanisms involved. Especially, the enhanced HLA-ABC expression of unmatched *donor* origin on the homograft target cells (36) together with the predominance of T lymphocytes with the *recipient's* MHC restriction in T cell mediated cytotoxicity (type 1 or 2 of table II) offers explanatory problems.

The non-B non-T cell subsets (OKT1 – 8 +) contribute to the inflammatory infiltrate; here, the lack of correlation with circulating antibodies (anti-HLA, LMA) does not make the interpretation any easier than in IA-CALD. Further complicating factors are interfering viral infections and changes in the Kupffer cell population (36, 37). However, our study of homograft pathology did elucidate the lack of pathogenetic involvement of LMA in any type of CALD. These results also implied that the reappearance of serum LMA in patients after orthotopic liver transplantation is without any consequence for the follow-up in general and for reoccurrence of IA-CALD in particular.

CONCLUDING REMARKS

It should be evident from the foregoing that not all pathogenetic mechanisms of the tissue destruction in CALD have been unraveled completely. However, the results do allow for a reformulation of the problems involved, enabling new possibilities for further research. Some of the lines along which further research might be pursued include the following. With immuno-electron microscopy the in situ presence of immunoglobulins attached to hepatocyte surfaces in areas with PMN can be studied, as this might contribute to solve the problem whether or not immunoglobulins in vivo do participate in the cytotoxic reaction. With functional in vitro studies the properties of potential effector cell subsets in the liver can be evaluated, provided that they are used in an autologous model with shortly cultured hepatocytes (Ch. 6). For the study of the contribution of Kupffer cells and other sinusoidal cells to the immunomodulation and the pathogenesis of CALD, liver homograft pathology or an animal model of CALD seem best suited. At least, they seem to provide better possibilities than the material and methods used for this thesis, as no conclusive evidence of any participation of these cells could be obtained so far.

The practical applicability of the methods used in this thesis, is still rather limited for diagnostic purposes. In diagnostic histopathology of CALD, the proper use of routine histologic staining methods usually settles the problem. In an incidental case, phenotyping of lymphocyte subsets might be of some additional help. The determination of serum LMA titers may be of additional help in the differential diagnosis of CALD. However, the practical value of the methods used and the results obtained may become evident in clinical hepatology, as the understanding of pathogenetic mechanisms in liver diseases is fundamental for the design and interpretation of therapeutical regimens.

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Summary

The purpose of this thesis was to investigate, which immunological mechanisms may be important for liver cell damage in different types of liver diseases.

The common histological feature of piecemeal necrosis in CALD of different aetiology has suggested that the liver cell damage could be the result of one pathogenetic mechanism. The heterogeneity of the underlying causes of CALD is reflected by for instance the different course of the several diseases and the contrasting clinical response to therapy. Although it is known that immunological mechanisms may contribute to the pathogenesis of some types of CALD, these findings are mainly based on in vitro studies with lymphocytes from the peripheral blood.

However, it is necessary to study the liver in vivo, because major differences may occur between the situation in the peripheral blood and in the liver.

For example, in the peripheral blood of patients with PBC a reduced number of OKT8 + T cells was found, whereas in the liver an increased number of these cells was seen.

A short general introduction is given in *chapter 1.*

In *chapter 2* the inflammatory infiltrate in liver biopsies of patients with CALD was studied with monoclonal antibodies to surface antigens present on subsets of functionally characterized lymphocytes. Special emphasis was given to areas with PMN. In areas with PMN in IA-CALD OKT8 + and OKM + lymphocytes and IgG plasma cells were present, whereas in HB-CALD almost exclusively OKT8 + T cells were found. In PBC OKT4 + T cells in the central parts of portal tracts and OKT8 + T cells in areas with PMN predominated. These findings indicate that in IA-CALD an ADCC reaction, as well as T cell cytotoxicity may be responsible for liver cell damage, whereas in HB-CALD specific T cell cytotoxicity seems to be the only mechanism. In PBC liver cell damage also predominantly is the result of T cell cytotoxicity. In addition, helper T lymphocytes seem to play a role, since these are found in central areas of the portal tracts.

In *chapter 3* the characteristics and distribution of the inflammatory infiltrate in liver biopsies of patients with hepatitis B viral infection were studied in relation to the distribution and expression of HBV antigens. Although in HB-CALD a T cell cytotoxicity appears to be the only immune mecha-

nism, in acute hepatitis B OKT8 + cells of non-T origin (OKT1 –, 3 –) and Leu7 + cells with presumed natural killer potential predominated in areas with liver cell necrosis, and non-T cell cytotoxicity appears to be the predominant immune mechanism. In none of the disease entities a positive spatial relation could be established between the cytotoxic cells and the demonstrable expression of HBV antigens in hepatocytes. It is concluded that differences in immunological reaction pattern may explain the different course in the forms of HBV infection.

In the last part of chapter 3 it is pointed out that the phenotype of a given lymphocyte subset may rather be judged by the application of a panel of monoclonal antibodies than by a limited selection.

In *chapter 4* serial graft biopsies from liver transplant recipients were studied with the use of histology, histochemistry, immunostaining, and electron microscopy. Planned protocol needle biopsies were taken from the graft before removal from the donor, 1 hour after transplantation, on the eighth day, and at yearly intervals. Nonprotocol biopsies were taken when deterioration of the clinical condition made a decision on changes in the regimen necessary. The protocol biopsies provided a baseline for graft condition and diagnostic histopathologic features. In these biopsies signs of hyperacute rejection, chronic rejection, or the recipient's previous liver disease were not observed. Mild acute rejection was regularly present on the eighth day, possibly due to a lag phase in the effect of immunosuppression. The syndromes in the nonprotocol biopsies included "pure" parenchymal cholestasis, reversible acute rejection resembling chronic active hepatitis, viral infection, and acute bacterial cholangitis. Each of these syndromes correlated with a separate histopathologic entity. Therefore, these entities proved to be of diagnostic value. It is concluded that a graft biopsy may substantially add to the pathogenetic interpretation, differential diagnosis, and management of major graft syndromes in orthotopic liver transplant recipients.

in *chapter 5* the mononuclear cell infiltrate in liver biopsies of patients with OLT has been studied. It has been found that in rejection the inflammatory infiltrate in the liver resembles both IA-CALD, with many plasma cells and OKT8 + lymphocytes in the areas with PMN, and PBC with many OKT4 +/Leu 3+ lymphocytes in the central parts of the portal tracts. In viral infection the picture was variable. It is concluded that liver cell damage in rejection is caused by T cell-mediated cytotoxicity.

In *chapter 6* liver membrane autoantibodies (LMA) have been studied. LMA has been thought to represent a pathogenetic factor for liver cell

necrosis by ADCC in immune mediated liver diseases. This presumed role of LMA has been studied by comparison of its presence in serum samples with the phenotype of potential effector cell subsets in liver biopsies taken at the same time in eight patient groups, including liver homograft recipients. Within the groups no difference in effector cell subsets was found between LMA + and LMA – patients. In the follow-up of individual patients before and during therapy, the presence of LMA and the phenotype and activity of the inflammatory infiltrate varied without any interrelation. It is concluded, that LMA and effector cell subsets in the liver are two independent variables, suggesting that LMA is presumably not a pathogenetic factor in the immune attack of the liver, but an epiphenomenon of the activated immune system.

In *chapter 7* the results of the preceding chapters are described in a general discussion.

Samenvatting

In dit proefschrift worden de resultaten beschreven van een onderzoek naar de immunologische mechanismen, die een rol zouden kunnen spelen in de beschadiging van levercellen bij verschillende soorten leverziekten.

Hoewel een chronische vorm van leverontsteking (chronisch actieve hepatitis) kan ontstaan door verschillende oorzaken (virussen, autoimmuniteit, geneesmiddelen), wordt histologisch vaak hetzelfde beeld gezien in de lever en daarom heeft men lange tijd verondersteld, dat één en hetzelfde mechanisme verantwoordelijk is voor het levercelverval (piecemeal necrose). Toch blijken er grote verschillen te bestaan tussen de diverse vormen van leverontsteking, wanneer bijvoorbeeld gekeken wordt naar het beloop van de ziekte en de reaktie op therapie. Hoewel bekend is, dat immunologische factoren een rol spelen bij levercelverval, is dit voornamelijk onderzocht door middel van in vitro studies aan lymfocyten uit het perifere bloed. Het is echter noodzakelijk om juist de lever „in vivo" te onderzoeken, aangezien er grote discrepanties kunnen voorkomen tussen de situatie in het perifere bloed en in de lever. Zo wordt bijvoorbeeld bij primaire biliaire cirrose in het perifere bloed een afname van een bepaald type lymfocyten gevonden, terwijl er in de lever juist een toename van deze cellen wordt gezien.

Een korte algemene introduktie wordt gegeven in *hoofdstuk 1.*

In *hoofdstuk 2* zijn leverbiopsieën van patiënten met chronische leverontsteking (chronisch actieve hepatitis) bestudeerd, waarbij het ontstekingsinfiltraat werd gekarakteriseerd met behulp van monoclonale antilichamen, welke gericht zijn tegen antigenen, die voorkomen aan het oppervlak van functioneel verschillende typen van lymfocyten. Speciale aandacht werd geschonken aan de gebieden met piecemeal necrose, dat wil zeggen, de gebieden waar levercellen ten gronde gaan.

Het blijkt, dat bij een chronische autoimmuun hepatitis voornamelijk OKT8 + en OKM + lymfocyten worden gezien naast IgG plasmacellen. Bij chronische hepatitis op basis van een hepatitis B virus infektie worden bijna alleen OKT8 + T cellen gevonden, terwijl bij primaire biliaire cirrose in de gebieden met piecemeal necrose OKT8 + T cellen worden gezien. Op grond van deze bevindingen lijkt het levercelverval veroorzaakt te worden door

zowel een antilichaam afhankelijke cellulaire cytotoxie, als een specifieke T lymfocyt cytotoxie in autoimmuun hepatitis en uitsluitend door een T lymfocyt cytotoxie in chronische hepatitis B en primaire biliaire cirrose. De helper T lymfocyten en plasmacellen in de portale velden in primaire biliaire cirrose zijn mogelijk van belang voor een humorale respons.

In *hoofdstuk 3* zijn patiënten met akute en chronische virale hepatitis B bestudeerd, waarbij speciale aandacht is geschonken aan de relatie tussen de verschillende typen ontstekingscellen en de expressie van virale antigenen in de levercellen. Hoewel in de chronische fase een specifieke T lymfocyt cytotoxie van belang lijkt te zijn, blijkt dat in de vroege fase van de akute hepatitis B juist voornamelijk NK cellen worden gevonden. Zowel bij de akute als chronische hepatitis werd geen direkte relatie gevonden tussen de aanwezigheid van virale antigenen in de levercellen en de cytotoxische effectorcellen. Geconcludeerd wordt, dat verschillen in het immunologische reaktiepatroon van de patiënt waarschijnlijk verantwoordelijk zijn voor het verschillend beloop van virale hepatitis B.

In hoofdstuk 3 wordt tevens gewezen op de noodzaak meerdere monoclonale antilichamen naast elkaar te gebruiken om de aard van de ontstekingscellen zo goed mogelijk vast te leggen.

In *hoofdstuk 4* zijn leverbiopsieën bestudeerd, waarbij de diverse morfologische beelden, die kunnen ontstaan in de lever na transplantatie, zijn vastgelegd. Hiervoor zijn leverbiopsieën onderzocht, die bij iedere patiënt genomen zijn vóór de transplantatie uit de donor lever, 1 uur na de operatie, op de 8e dag en na elk jaar. Daarnaast zijn biopsieën onderzocht, die genomen werden wanneer de klinische toestand van de patiënt dit noodzakelijk maakte.

In de geprotocolleerde biopsieën werden geen tekenen van hyperacute rejectie, chronische rejectie of de oorspronkelijke leverziekte gevonden. Geringe acute rejectie was regelmatig aanwezig op de 8e postoperatieve dag, mogelijk door een nog onvolledige instelling op de immunosuppressieve therapie.

De belangrijkste syndromen in de niet-geprotocolleerde biopsieën vormden acute rejectie en virale infekties, waarbij bleek, dat deze syndromen gekenmerkt worden door specifieke histologische beelden. Een juiste diagnose is van groot belang gezien de grote verschillen in therapie, respectievelijk een hogere en een lagere dosis immunosuppressie.

Geconcludeerd wordt, dat leverbiopsieën belangrijk zijn voor het te voeren beleid bij patiënten met een getransplanteerde lever.

In *hoofdstuk 5* is het ontstekingsinfiltraat gekarakteriseerd in leverbiopsieën van transplantatie patiënten met acute rejectie en/of virale infecties. Het beeld dat gevonden wordt bij acute rejectie lijkt enerzijds op autoimmuun hepatitis met veel plasmacellen en OKT8 + lymfocyten in de gebieden met piecemeal necrose, anderzijds op primaire biliaire cirrose met veel OKT4 +/Leu3 + lymfocyten centraal in de portale velden. Bij virale infecties was het beeld niet consistent. Het celverval bij acute rejectie lijkt voornamelijk veroorzaakt door een T lymfocyt gemedieerde cytotoxie.

In *hoofdstuk 6* is de rol van levermembraan autoantilichamen (LMA) onderzocht, aangezien van LMA wordt gedacht, dat dit antilichaam pathogenetisch van belang is voor het levercelverval. Deze veronderstelde rol van LMA is onderzocht door tegelijkertijd de aanwezigheid van LMA in het serum en de aard van de ontstekingscellen in de leverbiopsieën te bepalen bij acht groepen patiënten, waaronder patiënten na levertransplantatie. Binnen de diverse groepen werd geen verschil gevonden in effectorcelpopulatie tussen LMA + en LMA – patiënten. Bij de follow-up van individuele patiënten vóór en na therapie, werd geen correlatie gevonden tussen de aanwezigheid van LMA en de aard en activiteit van het ontstekingsinfiltraat in de lever. Het blijkt, dat LMA in het serum en de effectorcellen in de lever twee onafhankelijke variabelen zijn en dat LMA eerder een uiting is van een geactiveerd immuunsysteem, dan dat het pathogenetisch van belang lijkt te zijn.

In *hoofdstuk 7* worden de resultaten van de voorafgaande hoofdstukken in relatie tot de literatuur in een algemene discussie besproken. Tenslotte worden mogelijkheden aangegeven voor verder onderzoek naar de pathogenese van chronisch actieve hepatitis.